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Accumulation of fructose-1,6-diphosphate in Non-Growing Staphylococcus aureus

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ACCUMULATION OF FRUCTOSE-1,6-DIPHOSPHATE IN NON-GROWING *STAPHYLOCOCCUS AUREUS*

by

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VITA

Charles Frederick Huettner was born in 1947 in Chicago, Illinois. After attending a college preparatory school in Chicago, he graduated from Roosevelt University in 1966. He spent one year at the University of Illinois at Chicago, and then returned to Roosevelt University, where he obtained a Masters degree in Microbiology in 1970. After beginning studies at the Loyola University Medical Center in Maywood in 1970, he spent four months on active duty in the Naval Air Reserve, returning to Loyola in August of 1971.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monosphosphate
AS	ammonium sulfate
ATP	adenosine-5'-triphosphate
2DG	2-deoxy-D-glucose
DHAP	dihydroxyacetone phosphate
DTT	dithiothreitol
EAA	essential amino acids
EDTA	ethylenediaminetetraacetic acid
EM	Embden-Meyerhof
ENZ	enzyme
FDP	fructose-1,6-diphosphate
FDPase	fructose-1,6-diphosphatase
F6P	fructose-6-phosphate
g	gram
GAP(D)	glyceraldehyde-3-phosphate (dehydrogenase)
αGDH	α-glycerol-3-phosphate dehydrogenase
G6P(D)	glucose-6-phosphate (dehydrogenase)
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMP	hexosemonophosphate
HPr	heat-stable protein
K-Pi	potassium phosphate
LDH	lactate dehydrogenase
M	molar
2ME	2-mercaptoethanol
mg	milligram
min	minute
(μ)mol	(micro)mole
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NADH	reduced nicotinamide adenine dinucleotide
NEAA	non-essential amino acids
NBT	nitro blue tetrazolum; 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride
ONPG	ortho-nitrophenyl galactoside
PBG	phosphate-buffered glucose
PEP	phosphoenolpyruvate
PFK	phosphofructokinase (fructose-6-phosphate kinase)
6PG(D)	6-phosphogluconate (dehydrogenase)
PGI	phosphoglucose isomerase
PGK	phosphoglycerate kinase
Pi	inorganic (ortho) phosphate
PK	pyruvate kinase
PMS	phenazine methosulfate
PTS	phosphotransferase system
Q_{O_2}	respiratory rate
RQ	respiratory quotient

LIST OF ABBREVIATIONS (cont'd)

TCA	tricarboxylic acid
TCA ACT.	tricarboxylic acid cycle activity
TEA	triethanolamine
TPI	triosephosphate isomerase
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
TSA	Trypticase Soy agar (BBL)
TSB	Trypticase Soy broth (BBL)
U	unit (of enzyme activity)
VFC	Vitamin-Free Casitone (Difco)
VFCA	Vitamin-Free Casamino Acids (Difco)
wt	weight

INTRODUCTION

The various mechanisms controlling metabolism in growing cells have been established for a number of microorganisms. These mechanisms include regulation of the amount and the activity of enzymes. Regulation of enzyme activity includes both negative feedback inhibition, common in anabolic sequences, and precursor activation (feed-forward), where an intermediate activates an enzyme distal in the pathway, such as the activation of pyruvate kinase in the glycolytic Embden-Meyerhof (EM) pathway by fructose-1,6-diphosphate (FDP).

Less well understood are the factors influencing the alternate pathways for an intermediate. For example, a major branch point in glucose catabolism occurs at glucose-6-phosphate, which may be metabolized by either the EM pathway or the hexosemonophosphate (HMP) pathway. Because these pathways have different metabolic functions, it is important to determine the factors that regulate the relative and absolute amounts of glucose traversing each pathway under various conditions.

The problem of the distribution of glucose between the EM and HMP pathways has been investigated by others in streptococci. It was observed that the in vitro activity of 6-phosphogluconate dehydrogenase in the HMP pathway was inhibited by the EM intermediate FDP. It was suggested that the same control operated in vivo and thus accounted for the homolactic fermentation of glucose via the EM pathway in these organisms.

In contrast to growing cells, comparatively little is known about the controls in non-growing (non-replicating) cells. Would the same controls operate or would they be replaced by new controls as a response to the

change in the metabolic requirements of the organism? Would such a drastic change from growth to non-growth cause a loss of control, resulting in alterations in the pattern of pathways or in the levels of intermediates?

This investigation was initiated to examine these phenomena in Staphylococcus aureus. This organism possesses both the EM and HMP pathways, which operate simultaneously. The oxidative portion of the HMP pathway is more variable than the EM pathway, both in percentage and in absolute amount of glucose catabolized. The activities of the pathways in S. aureus and in almost all other microorganisms are usually estimated in non-gowing cells. Therefore, this investigation attempted to answer three questions: (1) What are the activities of the EM and HMP pathways in non-growing cells under various conditions? (2) Do the levels of any glycolytic intermediates change significantly when growing cells are subsequently incubated under non-growing conditions? (3) Do any of the intermediates influence the activity of the EM and/or HMP pathways?

REVIEW OF THE LITERATURE

A. Carbohydrate metabolism of S. aureus

1. Pathways of carbohydrate metabolism

One of the earliest studies on carbohydrate metabolism in S. aureus was by Friedemann (88), who measured lactic, formic, and acetic acids, and alcohols produced during semi-aerobic growth in a beef-infusion peptone medium supplemented with glucose. Later, Friedemann (89) quantitated the products of glucose metabolism in unshaken, semi-aerobic cultures, and found two main reactions for glucose. One was conversion of 77-91% of the glucose to lactic acid, and the second involved further splitting of 2-17% of the three-carbon intermediates to ethanol and carbon dioxide. Formate and acetate were also found. Probably the first study of the individual reactions of the Embden-Meyerhof (EM) pathway was that of Fosdick and Rapp (80), who used dried powders of S. aureus for in vitro assays of the intermediates generated after the addition of a known EM pathway intermediate. Hexosemono- and -diphosphates, triose phosphates, phosphoglyceric acid, pyruvic acid, and lactic acid were detected.

Evidence for the presence of the hexosemonophosphate (HMP) pathway was first suggested by Fusillo and Weiss (90), but the simultaneous operation of both the EM and the HMP pathways was found by Hancock (104), who examined the effects of streptomycin on S. aureus. In non-growing cells incubating in phosphate buffer plus labeled glucose, streptomycin caused a slight decrease in production of $^{14}CO_2$ from both glucose-1- and -6-^{14}C, but the actual participation of the pathways was not quantitated.

The work of Strasters and Winkler (283) was an important contribution to elucidation of the major pathways of carbohydrate metabolism in S. aureus. The quantitative conversion of glucose to L-lactic acid under anaerobic conditions in the presence of arsenite again demonstrated the presence of the EM pathway. The EM pathway enzymes glucosephosphate isomerase, phosphofructokinase, fructose-1, 6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and lactic dehydrogenase were demonstrated in cell-free extracts, the last two enzymes being more active in cells grown in the presence of glucose. In contrast, fructosediphosphatase was more active in cells grown without glucose. Measured manometrically, gluconate caused greater oxygen consumption and carbon dioxide evolution than ribose, suggesting that a complete HMP pathway was present. This was supported by the presence of the enzymes of the HMP pathway in cell-free extracts. Comparing cells grown in nutrient broth with or without glucose, it was demonstrated manometrically that oxidation of gluconate, ribose, lactate, acetate, several tricarboxylic acid (TCA) cycle intermediates, and a number of amino acids, was suppressed in the glucose-grown cells. The use of glucose-1-^{14}C and glucose-U-^{14}C indicated that both EM and HMP pathways functioned simultaneously, and after quantitating the labeled carbon recovered, it was estimated that at least 64% of the glucose was metabolized through the HMP pathway in cells grown without glucose, whereas in the glucose-grown cells, the activity of the HMP pathway decreased to about 47%. The uncertainty about the actual percentages arose because of the possible recycling of fructose-6-phosphate produced by the transaldolase-transketolase reactions in the non-oxidative segments of the HMP pathway. Fructose-6-phosphate can be

converted to glucose-6-phosphate (G6P) which reenters the HMP pathway. Significant recycling of this type can cause a lower than actual estimation of the activity of the HMP pathway, since the G6P can partially spare that formed from the exogenously-supplied labeled glucose. The phenomenon of recycling was discussed by Wood and Katz (326). Strasters and Winkler (283) also found no evidence for the presence of the Entner-Doudoroff pathway in cells grown with gluconate, nor did they find phosphoketolase, a key enzyme in a fourth major pathway of carbohydrate metabolism.

Quantitatively, the main products of aerobic glucose catabolism were acetate and carbon dioxide (283), with some lactate, as found by Gardner and Lascelles (92) in both growing and non-growing cells. Theodore and Schade (291) assayed the products of the anaerobic catabolism of glucose and found 73-94% lactate, 4-7% acetate, and small amounts of pyruvate.

2. Metabolism of pyruvic acid

Pyruvic acid may be metabolized by several different reactions, depending on the organism and conditions. Although it had been shown that glucose was converted to lactic acid under anaerobic conditions (89,283) and that this conversion was quantitative in the presence of arsenite (283), Krebs (163) demonstrated earlier that 2 molecules of pyruvate specifically underwent a dismutation to lactic and acetic acids plus carbon dioxide, the reaction representing an intermolecular oxidoreduction. The dismutation of pyruvate was stimulated up to 14-fold by yeast extract and also by purified vitamin B_1 (thiamine). The dismutation of pyruvate under anaerobic conditions was confirmed by Barron and

Lyman (7), who also found that the rate of dismutation, measured by CO_2 evolution, was greater than the rate of oxidation, measured by oxygen uptake. Diphosphothiamine, the active form of thiamine, stimulated pyruvate oxidation in non-growing S. aureus by 38%.

Fosdick and Rapp (80) also studied pyruvate metabolism, but used a cell-free system. They could not detect formation of pyruvate from 3-phosphoglyceric acid under anaerobic conditions, but assumed that pyruvate was formed and reacted rapidly. Examining the reactions of pyruvate, no lactate was formed aerobically, but anaerobically there was a quantitative conversion to lactate. Strasters and Winkler (283) also observed the anaerobic dismutation of pyruvic acid, and under aerobic conditions, pyruvate was oxidized to acetate and carbon dioxide by the non-growing cells. The amount of CO_2 exceeded that of acetate in cells grown without glucose, suggesting that some of the acetate was oxidized by the TCA cycle.

Watt and Werkman (313) compared the pyruvate metabolism of S. aureus grown in a peptone-yeast extract medium with or without glucose. Cells grown without glucose maintained a high pH (around 7.4) and carried out a dismutation of pyruvate to acetate, lactate, and CO_2, whereas glucose-grown cells caused a decrease from pH 8 to pH 5, and converted pyruvate to acetylmethylcarbinol, acetate, and CO_2. The acetylmethylcarbinol-forming system was stimulated by diphosphothiamine, but the dismutating system was not.

Although pyruvate kinase was proposed to be the rate-limiting enzyme in the EM pathway of S. aureus by Bluhm and Ordal (15), it has not been studied in detail. However, this enzyme from a number of microbial and mammalian sources has been shown to be activated by the EM pathway

intermediate fructose-1,6-diphosphate (FDP) (95,119,149,180,185,314). Such regulation by an intermediate of an enzyme distal in the pathway is called precursor activation or feed-forward activation, in contrast to feedback inhibition (119).

Lactate dehydrogenase (LDH) occurs in S. aureus. The effect of growth conditions on activity (42,93) and the presence of isozymes (276, 277) have been examined. The activity of LDH from a number of bacteria (100,210,325) is dependent on FDP. In a survey of 130 strains of staphylococci, Schleifer and Kocur (255) found that the FDP-activated LDH occurred only in 21 strains of S. epidermidis, and not in S. aureus.

3. The tricarboxylic acid cycle and effects of glucose

One of the earliest suggestions of the presence of the Krebs tricarboxylic acid (TCA) cycle in staphylococci was the observation of Smyth (271), who studied the effects of vitamin B_1 (thiamine) on pyruvate metabolism in S. aureus. Measurement of pyruvate dismutation in non-growing cells indicated that succinate, fumarate, and malate were formed when pyruvate or oxaloacetate were added to the incubation mixture. Addition of thiamine stimulated pyruvate dismutation by 69% in cells grown in a vitamin-deficient synthetic medium.

Stedman and Kravitz (274) provided further evidence for a TCA cycle in S. aureus by measuring the "sparking" of pyruvate oxidation by succinate and fumarate, and of acetate oxidation by succinate, fumarate, α-ketoglutarate, L-malate, and oxaloacetate. From the similarity of the sparking patterns of pyruvate and acetate oxidation by known intermediates, and from similar amounts of inhibition of pyruvate and acetate oxidation by selective inhibitors (fluoride, selenite, malonate, and

arsenite), they concluded that both pyruvate and acetate were oxidized by a common route, most likely the TCA cycle.

Most of the enzymes of the TCA cycle have been identified in S. aureus: aconitase (136), isocitrate dehydrogenase (42,136), α-ketoglutarate dehydrogenase (223), succinic dehydrogenase (42,196,283), fumarase (283), and malate dehydrogenase (283). Evidence for the presence of citrate synthase (condensing enzyme) has also been presented (94).

An early observation on the effects of glucose on the metabolism of S. aureus was that of Sevag et al. (263), who found that cells grown in glucose broth were devoid of carboxylase activity (the anaerobic evolution of CO_2 from pyruvate), whereas carboxylase was present in cells grown in plain broth. Addition of cocarboxylase (thiamine pyrophosphate or diphosphothiamine) stimulated carboxylase activity by 50%. Sevag and Swart (264) examined the effects of several substances during growth in the presence of glucose on the subsequent oxidation of pyruvate, and found that methionine, cysteine, glutathione, thiamine, adenylic acid, or diphosphothiamine could not overcome the inhibitory effects of glucose in the medium. Measurement of the ability of cells to oxidize pyruvate during growth in the presence of glucose showed that pyruvate was oxidized early, when glucose utilization was at a minimum.

Changes in the oxidative ability of S. aureus during growth were also observed by Powelson et al. (236), who found that 6 h cells (grown without glucose) accumulated acetate in the presence of glucose or glycerol under non-growing conditions, but 10 h cells oxidized these carbohydrates completely to CO_2 and water. During growth of the cells, the pH of the medium decreased from an initial level of pH 7.4, and then rose to a final pH of 8.6-8.8 at 24 h. The drop in pH was caused by the

formation of acetic acid, which was detected in the medium. Goldschmidt and Powelson (97) examined the effects of age of cells and pH on oxidation of acetate and found that the presence of glucose in a synthetic growth medium inhibited acetate oxidation.

In studies on the effect of growth conditions on the oxidative ability of S. aureus, Collins and Lascelles (42) found that after growth of cells in the presence of glucose, non-growing cells could not oxidize acetate, succinate, or malate. The glucose-grown cells also had greatly reduced activity of succinate dehydrogenase, and malate dehydrogenase was undetected. Strasters and Winkler (283) found that there was low or no oxidation of citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, or oxalacetate in cells grown with glucose, whereas these intermediates were oxidized either slowly or at 4-5 times the endogenous rate in cells grown without glucose. The activity of succinate dehydrogenase was lower, and that of fumarase was undetected, in the cells grown with glucose.

Ivler (136) compared the effects of growth in glucose on coagulase-positive and coagulase-negative strains of S. aureus. The ability of non-growing cells of both types to oxidize acetate, citrate, α-ketoglutarate, succinate, fumarate, and malate decreased to zero after the cells were grown with glucose. After growth with glucose, both coagulase-positive and -negative cells had little or no aconitase or isocitric dehydrogenase activity.

These effects of glucose on the synthesis of enzymes in S. aureus resemble the phenomenon of catabolite repression, also known as the glucose effect, which has been examined in E. coli (35,181,222,226).

Although catabolite repression has not been studied in staphylococci, various aspects of cellular metabolism have been implicated in this phenomenon in E. coli, including products of glucose catabolism (209), products of pentose or triose metabolism (177), redox systems (40,60), glucose-6-phosphate (131), and N-acetylglucosamine (61,62). The cyclic nucleotide adenosine-3',5'-monophosphate (cyclic AMP) has been intensely studied ever since Makman and Sutherland (183) observed a 32% decrease in the intracellular level of cyclic AMP in E. coli incubating in phosphate buffer. The addition of glucose to the incubation medium caused a 99% decrease in the level of cyclic AMP in the cells. The relationship between cyclic AMP and catabolite repression was recognized when Perlman and Pastan (225) found that cyclic AMP could overcome the repression of β-galactosidase synthesis caused by glucose. The mechanism of the glucose effect in S. aureus is yet to be elucidated.

4. Metabolism of carbohydrates other than glucose

Staphylococci are able to metabolize a number of hexoses other than glucose. One of the characteristics used to separate S. aureus from S. epidermidis is the utilization of mannitol. Murphey and Rosenblum (206) found that enzymes for mannitol metabolism were induced when S. aureus was grown in the presence of mannitol, glucitol (sorbitol), or arabitol, although only mannitol was metabolized. Growth in the presence of mannitol allowed the highest rate of uptake of mannitol, while glucose, glycerol, mannose, and fructose allowed the slowest rate, less than half of that with no carbohydrate. This inhibition was attributed to catabolite repression. The low, but detectable, activity of a nicotinamide adenine dinucleotide (NAD)-linked mannitol-1-phosphate dehydro-

genase in uninduced cells increased about 17-fold when cells were grown in the presence of mannitol. They presumed that mannitol-1-phosphate was an intermediate in mannitol catabolism, but no mannitol phosphorylating enzyme could be detected (206). The induction of mannitol-1-phosphate dehydrogenase was also observed by Strasters (282), who also found that in cells grown with mannitol and then incubated under non-growing conditions in the presence of mannitol, the level of mannitol-1-phosphate accumulated about 5-fold compared to cells grown with mannitol and incubated with glucose or fructose. Strasters (282) demonstrated by paper chromatography the formation of a compound corresponding to mannitol-1-phosphate by cells grown and incubated with mannitol, but he could not detect mannitol kinase activity in cell extracts, although such activity was inducible. The problem of the elusive mannitol kinase was solved when Simoni et al. (268) isolated a mannitol-specific and mannitol-induced factor III as part of the phosphoenolpyruvate-phosphotransferase system of S. aureus. Edwards and Blumenthal (personal communication) have recently identified free mannitol as an intracellular product in staphylococci and demonstrated that it is formed from glucose.

Chapman (39) showed that lactose, as well as glucose, fructose, glycerol, galactose, mannose, maltose, and trehalose, were fermented by pathogenic and by most non-pathogenic staphylococci. Hengstenberg et al. (112) found that a number of carbohydrates, lactose included, occurred as the phosphorylated derivatives intracellularly. Bissett and Anderson (11,12) elucidated the pathway of lactose metabolism in S. aureus. The phosphorylated lactose was hydrolyzed to glucose and galactose-6-phosphate by a 6-phospho-β-D-galactosidase (116). The galactose-6-phosphate was then isomerized to tagatose-6-phosphate and a

kinase formed tagatose-1,6-diphosphate, which was cleaved to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate by an aldolase distinct from FDP aldolase. Thus, this pathway is different from the Leloir pathway, where galactose is converted to glucose-6-phosphate (147).

The utilization of a large number of carbohydrates by staphylococci has been used in the classification of these organisms. From the initial efforts of Baird-Parker (7,8), who arranged the staphylococci into six subgroups, the problem was compounded by Kloos and Schleifer (152,153,256), who have proposed names for no less than ten species of staphylococci.

5. Electron transport and respiration in staphylococci

Aerobically-grown S. aureus possesses an electron transport system in the cytoplasmic membrane, along with a large fraction of succinic, lactic, malic, formic, α-glycerophosphate, and glucose-6-phosphate dehydrogenases (196). Taber and Morrison (286) examined the electron transport system of S. aureus in detail and identified three hemoproteins in a particulate membrane fraction: cytochrome a, cytochrome b1, and cytochrome o. The postulated scheme for electron transport was: NADH or succinate → flavoprotein → light-sensitive component → cytochrome b → cytochrome a → cytochrome o (cytochrome oxidase) → oxygen. Frerman and White (87) found that a shift from anaerobic to aerobic conditions during growth of S. aureus resulted in a 15-fold increase in the cytochrome a content and a 55-fold increase in the cytochrome o content of the cells. The electron transport chain and a number of oxygenases and dehydrogenases were recently shown to be associated primarily with protoplast membranes instead of mesosomal vesicles (292).

The repeated observations on the reduced oxidation of lactate, acetate, and intermediates of the TCA cycle after growth of S. aureus in the presence of glucose (42,136,236,283) have been explained by decreased activities of succinic dehydrogenase, isocitric dehydrogenase, and fumarase (42,283), but Strasters and Winkler (283) found a 40% reduction in the cytochrome content of S. aureus grown with glucose.

In addition to oxygen, staphylococci can use nitrate (NO_3^-) as a terminal electron acceptor (37,38,173). This process is called dissimilatory nitrate reduction (74).

6. Nutritional requirements of staphylococci

Staphylococci are heterotrophic organisms requiring a number of preformed amino acids and nicotinic acid (or nicotinamide) for growth. In 1932, Hughes (133) discovered a substance in meat extract that stimulated the growth of staphylococci in a synthetic medium. Knight (154) confirmed the existence of this "staphylococcal growth factor" isolated from yeast extract (marmite) which allowed aerobic growth in a medium of acid-hydrolyzed gelatin plus glucose, tryptophan, tyrosine, and cystine. Fildes et al. (77) developed a synthetic medium consisting primarily of amino acids, glucose, phosphate, and the "staphylococcus growth factor." This allowed aerobic growth, but for anaerobic growth, pyruvic acid and a "factor III" were necessary. Richardson (242) found that "factor III" was uracil and Knight (156) identified the growth factor as nicotinic acid and vitamin B_1 (aneurin). Nicotinamide was more effective than nicotinic acid and nicotinamide and vitamin B_1 (thiamine) were effective at very low concentrations, 2.5×10^{-8} M and 4×10^{-10} M, respectively (155). Thus, it became possible to grow

staphylococci in a completely defined, synthetic medium.

Knight (155) found that nicotinamide adenine dinucleotide (NAD, cozymase) could be replaced by nicotinic acid (or nicotinamide) as part of the "staphylococcal growth factor," thus demonstrating that nicotinamide was the constituent of NAD required as a vitamin for staphylococci. The metabolic role of NAD and NADP in oxidation-reduction reactions was established by Warburg and Christian around 1934.

S. aureus was originally considered able to utilize only nicotinic acid or nicotinamide (157), but Wadke (306, 308) has shown that these can be replaced by a number of niacin analogs that are fully effective, but only at much higher concentration, viz., mg instead of µg per ml.

The story of thiamine began with Auhagen (161), who discovered cocarboxylase, a dialyzable factor necessary for the activity of carboxylase, which converts pyruvic acid to acetaldehyde and CO_2. The active form of cocarboxylase was found to be a pyrophosphoric ester by Lohmann and Schuster (161) in 1937. The name thiamine was first applied in 1937; thus, the active form is known as diphosphothiamine (DPT) or thiamine pyrophosphate (TPP). The role of thiamine in pyruvate metabolism of staphylococci was determined by Hills (121).

Studies on the amino acid requirements of staphylococci probably began with Gladstone (96) in 1937, who grew 26 clinical isolates of staphylococci (primarily S. aureus) in a synthetic medium containing 16 amino acids. Fildes and Richardson (76) found that the requirement for a sulfur source could be satisfied by a number of mercapto- or dithio- compounds, but cystine was far superior to any of them, being effective at a concentration of 10^{-6} M. The amino acids generally

considered to be essential for staphylococci include arginine, cysteine, proline, and valine (73,166). Most recently, nutritional mutants of S. aureus H were grown in media in which the only amino acids were arginine, cysteine, glutamic acid, and proline (3).

The vitamin requirements and general nutrition of staphylococci are discussed in more detail by Koser (161) and Elek (72), respectively.

7. Regulation of the EM and HMP pathways

Regulation of metabolism occurs on two general levels: one, on the synthesis of enzymes, resulting in changes in their type and/or amount; two, on the activity of enzymes, which may be caused by availability of substrates and cofactors, and may result in a change in the overall rate of a pathway, particularly if the enzyme is rate-limiting for the pathway. Most of the research on staphylococci has been on the second type of control, and the approach usually was to vary the growth conditions and observe resulting changes in pathway activity, end products, or enzyme levels. There have been few studies on isolated enzymes.

An early study that established the roles of niacin (hence of NAD) and thiamine in S. aureus was that of Kligler et al. (151), who found that niacin was absolutely essential for utilization of glucose and pyruvate under both aerobic and anaerobic conditions. In the presence of niacin and thiamine aerobically, glucose was converted to lactate (20%), acetate (40%), a trace of pyruvate, and presumably the remainder was CO_2. Elimination of thiamine yielded 60% lactate and 40% pyruvate. Therefore, niacin was essential for glycolysis and thiamine was involved in the further oxidation of pyruvate. Anaerobically, added thiamine was unnecessary (the complex medium probably already contained low

levels), but pyruvate was essential for growth, whether produced glycolytically or added exogenously. No growth occurred without niacin. Glucose was converted primarily to lactate, and pyruvate underwent dismutation to lactate, acetate, and CO_2 (151).

Hughes (132) found that niacin (nicotinic acid) stimulated glycolysis by about 80% in suspensions of non-growing S. aureus. After adapting a strain of S. aureus to grow on the thiamine analog pyrithiamine instead of thiamine, Das and Chatterjee (49) found decreased glucose oxidation, but greater acetate oxidation, by the adapted strain. The ratio of $^{14}CO_2$ from glucose-1-^{14}C to that from glucose-6-^{14}C was about 1.9 in the parent strain and 0.8 in the adapted strain. This result, combined with the decreased glucose oxidation, suggested that significant changes in operation of the HMP pathway, and also to some extent in the EM pathway, had occurred.

The use of radioisotopes provided greater capability for investigation of pathways and of the effects of inhibitors, analogs, etc. This approach was used by Blumenthal et al. to measure the actual in vivo participation of the pathways in S. aureus under various conditions. Montiel and Blumenthal (199) found that addition of glucose to the Trypticase growth medium caused a reduction of the activity of the HMP pathway from 34% to 26%, and a greater reduction of the activity of the TCA cycle from 17.0 to 0.5, measured in non-growing cells incubating in the presence of C_1- or C_6-labeled glucose. Blumenthal et al. (19) found that addition of 1 mg of thiamine per liter of Trypticase broth caused a 20- to 50-fold increase in activity of the TCA cycle and a decrease in activity of the HMP pathway. Addition of 2-4 mg of nicotinic acid per liter of Trypticase did not significantly affect the TCA cycle, but

stimulated the HMP pathway 40-50% (19).

A brief explanation of the nature of the media used is in order. Peptone refers to the diffusable peptides resulting from the partial hydrolysis of protein from plant or animal sources. Tryptone is an enzymatic digest of casein with a high concentration of tryptophan, suitable for the determination of indole production. Trypticase is a trade name (BBL) for a tryptone derived from casein. Casitone is a trade name (Difco Laboratories) for a pancreatic digest of casein.

To more effectively control the vitamin levels during growth of S. aureus, Hoo et al. (128) employed a "vitamin-free" Casitone without the addition of glucose, which had been shown to inhibit both the HMP pathway and the TCA cycle (199,283). They found about 6% HMP pathway and low levels (0.1-0.6 μmol/g dry wt) of the nicotinamide coenzymes in cells grown in unsupplemented medium. Addition of niacin increased the HMP pathway to around 20% and caused a 10-fold increase in the NAD concentration (128). There were also 2- to 3-fold increases in the activities of G6P and 6PG dehydrogenases in the niacin-supplemented medium. Effects of niacin on metabolism in suspensions of non-growing S. aureus were observed by Wadke et al. (307). Cells grown without niacin were incubated in phosphate-buffered glucose. Upon addition of niacin, NAD and NADP levels increased by factors of 15 and 2, respectively, and the HMP pathway was stimulated 3-fold in the non-growing cells, even in the presence of 100 μg of chloramphenicol/ml to preclude protein synthesis. In a continuation of this work by Hoo et al. (127), S. aureus was incubated in phosphate-buffered glucose for 8 h, and both pathways and NAD(P) levels were measured. Initially, the activity of the HMP pathway was about 18%, with 2.4 μmol NAD/g dry wt. After 6 h,

the HMP pathway had decreased to 8% and NAD to 0.6 μmol/g. At this time, niacin was added to the suspension of non-growing cells to a final concentration of 100 μg/ml, and after 2 additional h of incubation, the HMP pathway increased to 16% and NAD to 1.2 μmol/g. During the entire 8 h, the activity of the TCA cycle remained relatively constant, and NADP, which had also remained constant, increased from 0.5 to 0.8 μmol/g after addition of niacin (127). This seemed to suggest that NAD, and not NADP, was controlling glucose catabolism through at least the oxidative portion of the HMP pathway. This variability of the HMP pathway from about 6% to more than 20% has been called the "expandable portion" of the HMP pathway by Blumenthal (17), who also showed that maximum growth of S. aureus in thiamine-supplemented Vitamin-free Casitone was obtained with 0.05 μg niacin/ml and about 11% HMP pathway. Higher concentrations of niacin, up to 0.25 μg/ml, caused no increase in growth of the organisms but allowed HMP pathway activity to increase to 22%. Higher niacin concentrations, up to 50 μg/ml, during growth caused no further increases in either growth or HMP pathway activity. Measurement of NAD levels with increasing niacin concentrations during growth of S. aureus resulted in a 19-fold increase to a maximum of 5.7 μmol/g at 2.0 μg niacin/ml. In contrast, NADP underwent only a 3-fold increase, to a maximum of 0.6 μmol/g at 2.0 μg niacin/ml (17). Again, this suggested a relationship between NAD levels and the activity of the HMP pathway. This problem was at least partially solved by Montiel et al. (200), who discovered isozymes of G6P and 6PG dehydrogenases that could use either NAD or NADP as coenzyme, if the NAD concentration was about 10 times higher than NADP. Therefore, the extent of glucose catabolism by the HMP pathway in S. aureus can be varied by glucose and

niacin in the growth medium.

The EM and HMP pathways were also affected by the pH of the growth medium. Using a vitamin-supplemented casein hydrolysate medium, Morse (203) found greater EM pathway activity in the presence of glucose or at pH 7.7, and greater HMP pathway activity in the absence of glucose or at pH 5.6.

Bluhm and Ordal (15) examined the effects of sublethal heat on the activity of the glycolytic enzymes of S. aureus in cell-free extracts. Only the activities of fructosediphosphate (FDP) aldolase, LDH, and butanediol dehydrogenase were reduced significantly as a result of heating the whole cells at 52C. Pyruvate kinase had the lowest activity, suggesting that it was the rate-limiting enzyme in the EM pathway. The activity of glucose-6-phosphate dehydrogenase was considerably lower than that of 6-phosphogluconate dehydrogenase, suggesting that the former may be rate-limiting in the oxidative portion of the HMP pathway. Bluhm and Ordal (15) also estimated pathways for glucose oxidation in non-growing cells and found 88.7% EM and 11.3% HMP in normal, unheated cells, and 89.5% EM and 10.5% HMP in heat-injured cells.

In studies of the regulation of the HMP pathway, most workers appear to have been concerned with the factors regulating the in vitro activities of glucose-6-phosphate dehydrogenase (G6PD) and/or 6-phosphogluconate dehydrogenase (6PGD). The results were then usually extended to the in vivo situation. Consideration of the factors that regulate the distribution of glucose carbon between the EM and HMP pathways would be a requisite of such studies, as G6P is common to both pathways.

Brown and Wittenberger (24) have investigated this problem in the

homofermentative organism, Streptococcus faecalis. Analysis of enzyme levels and the decrease of $^{14}CO_2$ evolution from non-growing, gluconate-adapted cells caused by unlabeled glucose suggested that 6PGD and not G6PD was the site of control of the HMP pathway in S. faecalis. A survey of most of the EM pathway intermediates showed that only FDP caused inhibition of the NADP-linked 6PGD in vitro. This was also true for 6PGD from all other sources tested, including other streptococci, E. coli, Candida utilis, and guinea pig liver. Based on this in vitro effect of FDP on 6PGD and meager in vivo data, they postulated a scheme whereby FDP would inhibit 6PGD and activate LDH (324), thus causing a homolactic fermentation under conditions where adenosine 5'-triphosphate (ATP) levels would presumably not inhibit PFK, allowing maintenance of a pool of FDP in these cells (24). The activation of LDH by FDP is well-established for streptococci (27,324,325), and was used by Schleifer and Kocur (255) as a criterion for classification of staphylococci, only the S. epidermidis enzyme being activated by FDP. Brown and Wittenberger (26) also found that glucose-grown Streptococcus faecalis contained a NADP-linked 6PGD, but growth in the presence of gluconate caused induction of a NAD-linked 6PGD as well. Based on the specific inhibition of the NADP-6PGD by FDP and of the NAD-6PGD by ATP, they postulated that the NADP-linked enzyme was involved primarily in production of NADPH for biosynthesis, and that the NAD-linked enzyme functioned in gluconate catabolism (26).

Study of regulation of the pathways in Gram negative organisms has centered on Escherichia coli and on Pseudomonas sp. These organisms differ in their oxygen requirements and in their glucose catabolic path-

ways, hence in their regulatory properties.

Model and Rittenberg (198) measured the HMP pathway in E. coli using glucose-1-^{18}O and found that about 25% of the glucose was metabolized by this route. The percent of glucose oxidized by the HMP pathway decreased as the cells entered the stationary phase, if the nitrogen source was exhausted, or under anaerobic conditions. Although neither enzyme activities nor coenzyme levels were measured, they postulated that the activity of the HMP pathway was controlled by the availability of NADP, since under the three conditions mentioned, the level of NADPH would be higher (198). An allosteric inhibition of G6PD by NADH was reported by Sanwal (254), who suggested that one of the major functions of the HMP pathway was to generate biosynthetic reducing power as NADPH. However, a later report (36) indicated that the observed inhibition of G6PD may have been an instrumental artifact.

A series of papers from the laboratory of Doelle yielded interesting results. In glucose-limited chemostat cultures of E. coli, the specific activity of PFK increased linearly as the oxygen partial pressure was lowered below 28 mm Hg (241). An expanded and extended study of the same phenomenon revealed that, in addition to PFK, the activities of G6PD, 6PGD, and FDP aldolase also increased with decreasing oxygen partial pressure (293). A significant finding was that PFK in aerobic cultures was not inhibited by ATP, but in anaerobic cultures (0 mm Hg), a second form of PFK was synthesized that was sensitive to inhibition by ATP (63,64). Because of the increased activity of FDP aldolase during the conversion to anaerobiosis, Thomas et al. (293) suggested that it may play a role in regulation of the EM pathway, although they presented no possible mechanism for such involvement.

Doelle et al. (66) also investigated the effects of glucose concentration on the enzymes of E. coli, grown aerobically and anaerobically. Aerobically, the activities of PFK and FDP aldolase underwent increases at 0.2% glucose and remained constant as the glucose concentration was increased to 1%. PFK was insensitive to ATP at all glucose concentrations. Anaerobically, FDP aldolase activity increased almost linearly with increasing glucose concentration (up to 1%), while PFK was most active at 0.08% glucose and was inhibited by ATP. Doelle et al. (66) also found that the anaerobic FDP aldolase yielded three peaks from a DEAE-Sephadex column, but the aerobic aldolase yielded only one. Under all conditions tested, the activities of G6PD and 6PGD remained about the same. These results suggested that there would be a minimum HMP pathway activity both anaerobically and aerobically, but that under anaerobic conditions, PFK would be inhibited by ATP. Therefore, under anaerobic conditions, both PFK and FDP aldolase were more active, and as aerobic conditions became established, these enzymes became less active, presumably inhibiting glucose utilization by the EM pathway and shifting more glucose into the HMP pathway. This is their explanation for the Pasteur effect (66), which is the inhibition of glucose utilization by oxygen, also referred to as the oxygen effect. The converse of this is the Crabtree effect, which is the inhibition of oxygen consumption by the utilization of glucose, also called the glucose effect or catabolite repression (65,66).

Westwood and Doelle (318) examined the properties of G6PD and 6PGD from E. coli. Assay of G6PD in the presence of ATP, AMP, FDP, NADPH, or NADH, showed that only NADPH had any inhibitory effect. All

microbial and mammalian enzymes assayed were inhibited by NADPH, and only the G6PD from Pseudomonas sp. and Acetobacter sp. were significantly inhibited by ATP. The E. coli 6PGD was inhibited by ATP, FDP, and NADPH, as were the other microbial and mammalian enzymes. Westwood and Doelle (318) also suggested that in E. coli growing aerobically, the FDP-mediated inhibition of 6PGD controlled the distribution of glucose between the EM and HMP pathways. However, no in vivo data regarding pathways or intermediate levels were given.

Szynkiewicz et al. (285) studied pentose biosynthesis in E. coli by measuring the relative specific activities of hexose and pentose carbons after growth of the cells on glucose or acetate followed by exposure to labeled glucose. Cells grown on acetate as the main carbon source synthesized most of their pentose via transketolase and transaldolase, i.e., the non-oxidative portion of the HMP pathway. During growth on glucose, however, the oxidative decarboxylation of 6PG predominated over the transketolase-transaldolase pathway. An extensive analysis of this problem was presented by Katz and Rognstad (148), who used published bacterial and mammalian data to demonstrate that the net flow of carbon was from hexose to pentose in the oxidative branch and from pentose to hexose in the nonoxidative branch.

Orthner and Pizer (221) compared two strains of E. coli, one a mutant with 10 times as much G6PD as the other, and found that the mutant had only 25% higher evolution of $^{14}CO_2$ from glucose-1-^{14}C and the same evolution of labeled CO_2 from glucose-6-^{14}C as the parent strain. Analysis of the intracellular levels of G6P and 6PG and the rate of CO_2 evolution from gluconate-1-^{14}C showed no significant differences between the strains, suggesting that G6PD was regulated and was rate-limiting.

The levels of nicotinamide coenzymes, oxidized and reduced, were also similar in the two strains. It was concluded that the factors regulating the activity of G6PD in vivo were substrate limitation or product inhibition. Therefore, in E. coli, 6PGD and not G6PD seems to be the regulatory enzyme in the HMP pathway, and its effectors include ATP, FDP, and NADPH.

As might be expected, the situation in Pseudomonas is different from that in E. coli. Eagon (68) surveyed a number of organisms with regard to pathways and the presence of NADPH oxidase and pyridine nucleotide transhydrogenase. Organisms using the HMP pathway and/or the Entner-Doudoroff (ED) pathway for a significant amount of glucose catabolism (20-50%) had levels of NADPH oxidase and transhydrogenase that were higher than those organisms with less than 15% HMP pathway activity. Pseudomonas aeruginosa had 29% HMP and 71% ED, and Ps. fluorescens had more than 50% HMP, 33% ED, and less than 17% EM, and both of these organisms had very high levels of NADPH oxidase and pyridine nucleotide transhydrogenase activities. Eagon suggested that the rate of the HMP pathway was limited by the supply of NADP, and that organisms with a significant percentage of HMP pathway activity could overcome this limitation by the presence of NADPH oxidase and transhydrogenase, as well as G6PD and 6PGD that could use either NADP or NAD (68).

We may generalize these findings regarding regulation of the pathways. In organisms with both EM and HMP pathways, 6PGD rather than G6PD appears to be the key regulatory enzyme for the HMP pathway because there are more effectors (ATP, FDP, NADPH) and the reaction tends to be irreversible. But the speculation regarding in vivo control of the pathways is based primarily on in vitro data from isolated and partially

purified enzymes. For the EM pathway, PFK seems to be the main regulatory enzyme, although FDP aldolase and GAPD may possibly function here also.

8. Carbohydrate transport into staphylococci

In 1964, Kundig et al. (164) reported the discovery of a phosphotransferase (PTS) system in E. coli. This system consisted of an enzyme I (Enz I), an enzyme II (Enz II), and a heat-stable protein (HPr) that were involved in the transfer of phosphate from phosphoenolpyruvate (PEP) to a histidine residue in the HPr, then to a hexose. The specificity of Enz II varied with the carbohydrate during growth, e.g., the Enz II for glucose differed from that for galactose. Enz I and HPr, however, were non-specific. Only PEP could donate phosphate and it could not be replaced by ATP or other nucleoside triphosphates.

Egan and Morse (69) reported the isolation of a pleiotropic, single gene mutant of S. aureus, designated car^-, which had lost the ability to utilize a number of carbohydrates, including fructose, lactose, mannitol, and sucrose, but which could still grow on glucose. A lack of uptake of sucrose, maltose, and glucose by car^- spheroplasts indicated that the cell wall was not essential for transport. Comparable rates of uptake of amino acids, vitamins, and some TCA cycle intermediates by mutant and parent strains disclosed that the car phenotype was involved only in carbohydrate transport (70). Further investigation of the car^+ strain showed that uptake of carbohydrates was saturable at 5 mM glucose and lactose and about 50 mM maltose, suggesting that permeases were involved. Lactose and α-methylglucoside accumulated intracellularly as derivatives, which were not identified (71).

The presence of a PTS in S. aureus, similar to that in E. coli, was first suggested by Kennedy and Scarborough (150), who found that the hydrolysis of o-nitrophenylgalactoside (ONPG) by S. aureus was inhibited by fluoride and was stimulated by PEP, but not by ATP, in acetone-treated cells. Early studies on lactose metabolism in S. aureus (48, 189) failed to detect-β-galactosidase in induced cells. The discrepancy between the whole cell and cell extract studies was resolved by Hengstenberg et al. (112, 113, 114, 116), who found that although intact cells could not hydrolyze lactose, it was taken up and phosphorylated.

The actual characterization of the staphylococcal PTS began with Simoni et al. (268), who demonstrated that the staphylococcal PTS was similar to that of E. coli, consisting of Enz I, HPr, and Enz II. However, S. aureus required an additional component, designated as Factor III, for the utilization of mannitol. Simoni et al. (268) also indicated that the car- mutant of Egan and Morse (69-71) was deficient in Enz I. Hengstenberg et al. (115) partially characterized each component of the staphylococcal PTS, using hydrolysis of ONPG for the assay. All four components were necessary for ONPG hydrolysis in an in vitro assay, and the uptake of isopropylthiogalactoside-2-^{14}C by a series of whole-cell mutants, each lacking one component, was negligible compared to the normal strain.

The lactose-specific, membrane-bound Enz II was solubilized by Hengstenberg (111, 160) and found to have a molecular weight of about 36,000. The lactose-specific Factor III had a molecular weight of about 33,000 (109, 115, 257). The molecular weight of Enz I was estimated to be 70,000-90,000 by Simoni et al. (266), and about 100,000 by Hengstenberg et al. (115). HPr was a relatively small protein with a molecular weight

of only 9,000-10,000 (109,115).

The actual route of phosphate transfer between the components has also been elucidated. The initial phosphorylation is of Enz I, not HPr (275). Phosphate is then transferred to HPr (266,268), then to Factor III (109,207). The final phosphate transfer, from Factor III to the sugar, is catalyzed by the membrane-bound, carbohydrate-specific Enz II (207,265). No enzyme is apparently required for the transfer of phosphate from HPr to Factor III (265).

The physiological role of the PTS in S. aureus was examined by Simoni and Roseman (267), using mutants to observe the effects on growth of the organisms. For example, mutants lacking Enz I could not ferment a variety of sugars, but Enz II-lac or Factor III-lac mutants (specific for lactose), exhibited specificity in their inability to transport thiomethylgalactoside, but could still transport methylglucopyranoside.

Therefore, the sugar transport mechanism of S. aureus is of the group translocation type, in contrast to that of E. coli, which does not involve phosphorylation of the sugar, hence is an example of active transport (105,142,143). The genetics of the galactoside transport system of S. aureus has been examined by Morse et al. (202), who indicated that apparently three genes control lactose metabolism: one for a phospho-β-galactosidase, one for a protein that concentrates phosphorylated galactosides, and one that controls the first two. They also indicated that galactose-6-phosphate was the best inducer of the lactose transport system.

There are at least two exceptions to the generalization that carbohydrates are transported and phosphorylated by a PEP-dependent PTS in staphylococci. Button et al. (31) found that maltose entered S. aureus

by diffusion and was then hydrolyzed to glucose and phosphorylated, possibly by some reactions of the PTS. Richey and Lin (243) demonstrated that glycerol was dissimilated by an ATP-dependent kinase, instead of a PEP-dependent phosphorylation by the PTS.

Winkler (323) recently demonstrated that S. aureus possessed an inducible hexose phosphate transport system, which transported G6P in the presence of the inducer, G6P. Such a system has been known for some time in E. coli (82,229) and has been examined extensively by Winkler (321,322) and by Dietz (56-59).

B. In vivo studies of non-growing cells

As may be induced from the previous section, the methods most commonly used to measure the participation of metabolic pathways, whether by the Warburg method (CO_2 evolution or O_2 uptake) or radiorespirometry ($^{14}CO_2$ evolution), employ non-growing ("resting," non-replicating) cells incubating in a buffer plus a carbohydrate, usually glucose, but without a nitrogen source. The lack of a nitrogen source implies that neither cell growth nor multiplication occurs, although the breakdown and resynthesis of protein is still possible. That such cells are not growing suggests that the system is not completely natural, and it would be reasonable to suppose that significant metabolic differences might exist between cells that ceased growth because a vital nutrient became exhausted, and cells that were prevented from growing by placement into a preclusive environment, whether lacking in a carbon or nitrogen source or both, or containing an inhibitory substance. This necessitates consideration of the endogenous metabolism of bacteria, followed by attention to the effects of the presence of a carbon source on endogenous metabolism.

1. Endogenous metabolism of S. aureus and other bacteria; effects of starvation.

Two terms used in these studies require definition. QO_2 is the respiratory rate, expressed as the volume of O_2 consumed per unit time per unit weight of organisms, i.e., μl O_2/h/mg or μl O_2/mg/h. The respiratory quotient (R.Q.) is the molar ratio of CO_2 produced to O_2 consumed, and has no units.

The endogenous metabolism of staphylococci was examined by Ramsey and by Ivler. Ramsey (239) observed that although the Q_{02} decreased as cells aged, the R.Q. remained constant. Cells grown in complex medium had a higher endogenous Q_{02} than cells grown in a defined medium. Several observations on the apparent lack of effect of glucose on endogenous metabolism were made. (a) Increased glucose concentration (from 0.5% to 7.5%) during growth resulted in only a slight increase in yield (from 19.6 to 26.4 mg dry wt/ml) and only a slight increase in endogenous Q_{02} (from 8.4 to 11.8). However, addition of Casamino acids during growth caused a 2-fold increase in Q_{02}, from 4.8 to 10.0. Lack of change in the total carbohydrate pool during starvation indicated that the endogenous substrate was not carbohydrate. Cells respiring endogenously liberated ammonia, part of which originated from the oxidation of glutamic acid. (b) The presence of glucose caused an initial inhibition of NH_3 release, but upon exhaustion of glucose, NH_3 release increased to ultimately equal that released in the absence of glucose. Cells grown with glucose-^{14}C, then incubated in the presence of unlabeled glucose or glutamic acid showed only slightly greater evolution of $^{14}CO_2$ than starved cells. (c) Exogenous glucose did not inhibit endogenous respiration.

Ivler (136) studied endogenous respiration, comparing coagulase-positive with coagulase-negative strains. The viability and dry weight of cells remained essentially constant during three hours of starvation in water or in phosphate buffer. The Q_{O2} for coagulase-positive cells was greater than that of coagulase-negative cells (10 vs. 4), and both decreased during starvation. Although the R.Q. of coagulase-positive cells remained essentially constant (decreasing from 1.08 to 0.95) during one hour of starvation, that of coagulase-negative cells decreased by about 50% (from 1.10 to 0.5). Glucose caused an increase in the Q_{O2} of coagulase-positive cells, but did not affect negative cells. Although total carbohydrate and lipid did not change significantly during starvation, the amino acid pools in coagulase-positive and -negative cells decreased by 41% and 27%, respectively. Whereas coagulase-positive cells released 47% of their pool glutamic acid, the more than 2-fold higher O_2/NH_3 ratio of coagulase-negative cells suggested that compounds other than amino acids served as substrates for endogenous metabolism. Coagulase-negative cells contained poly-β-hydroxybutyric acid, which decreased by 50% during starvation. The presence of glucose had no significant effect on amino acid pool depletion or on NH_3 release during incubation (136).

Therefore, in S. aureus, amino acids, especially glutamic acid, appeared to be important as endogenous energy sources, and the presence of glucose may have affected the rate of amino acid degradation (measured by NH_3 release) but did not decrease the total amino acids oxidized.

Similarities in the pattern of endogenous metabolism of Sarcina lutea were observed by Burleigh and Dawes (29). The rates of endogenous oxygen consumption and survival decreased in cells starved aerobically

in phosphate buffer, but cells survived more efficiently when glutamate or glucose was supplied exogenously. Sarcina oxidized intracellular amino acids, releasing ammonia and some ultraviolet radiation-absorbing material into the medium.

The effects of starvation of Streptococcus lactis in relation to survival were studied by Thomas and Batt (294,297), who found that amino acids, especially arginine, increased survival, but that fermentable carbohydrates caused accelerated death. Amino acids were released into the medium along with some soluble protein. There was no significant breakdown of carbohydrate (295). The addition of an energy source to starved S. lactis allowed limited protein synthesis, which depended on exogenous glucose. However, the recurrence of protein synthesis did not favor survival (296). Changes in permeability during starvation resulted in the release of lactate dehydrogenase and DNA into the medium, which was correlated with the ultimate loss of viability of the starved cells (298).

The effects of anaerobic starvation on Streptococcus faecalis were examined by Walker and Forrest (309), who found that carbohydrate, amino acids, and ultraviolet radiation-absorbing material were liberated by cells starved in phosphate buffer for 24 h. During 27 h of starvation, glycolytic activity decreased by 71%, but the presence of arginine or citrulline allowed glycolytic activity to decrease by only 17% and 27%, respectively. After reducing 93% of the glycolytic activity by starvation, the addition of Casamino acids or glucose increased glycolytic activity to 23% and 33% of the initial level, respectively, but a combination of Casamino acids plus glucose restored activity to 65% of the initial level. The major anaerobic endogenous substrates were amino acids, and maintenance of glycolytic activity depended on phosphate, which

implied that energy-linked reactions providing ATP were needed to maintain cellular organization. Further examination by Forrest and Walker (79) of anaerobic endogenous metabolism by S. faecalis revealed that ATP levels in starved cells suspended in phosphate buffer increased in the presence of glucose, but increased less in the presence of amino acids because of the synthetic reactions which utilized the ATP. Furthermore, the addition of amino acids to starved cells incubating in phosphate buffer plus glucose did not change the rate of glycolysis in these cells. They also found that cells with endogenous reserves (i.e., cells grown with sufficient glucose) possessed a constant ATP level for as long as constant glycolytic activity was maintained. Cells without endogenous reserves had no detectable endogenous metabolism, a decreasing ATP level, and decreasing glycolytic activity during starvation in phosphate buffer. They suggested that glycolysis in washed suspensions was not controlled by feedback, i.e., the presence of glucose and the generation of ATP did not affect the rate of glycolytic activity (79).

The effects of starvation on Gram negative organisms have been extensively investigated by Postgate and his collaborators. Postgate and Hunter (233) studied the survival of Enterobacter (Aerobacter) aerogenes in phosphate-buffered saline and found that the organisms died linearly with time. These organisms were grown in continuous culture with a limited supply of glycerol, and the presence of glycerol during incubation tended to accelerate the death of the organisms. Such acceleration of death by growth substrates was termed "substrate-accelerated death" by Postgate and Hunter (234) and occurred when organisms were grown with ammonium ion, phosphate, glucose, and mannitol as the limiting substrate, then incubated in buffered saline plus the respective compounds. Glucose-,

pyruvate-, and glycerol-accelerated death was observed with E. coli, Serratia marcescens, and E. aerogenes by Postgate and Hunter (235). A postulated mechanism for substrate-accelerated death involved repression of enzyme synthesis by the growth limiting substrate, but transfer of genetic information was probably more central to the problem.

Strange and Dark (278-280) confirmed the phenomenon of substrate-accelerated death in E. aerogenes. More importantly, glucose accelerated the death rate of bacteria grown with limiting amounts of glycerol, mannitol, galactose, and ribose. Strange (278) indicated that nitrogen-limited, glycogen-rich E. aerogenes and E. coli were able to survive starvation longer than glycogen-less organisms, implying that energy-yielding reactions were necessary for survival during starvation.

Calcott and Postgate (33) studied substrate-accelerated death in Klebsiella aerogenes and found that only the growth-limiting substrate, or metabolites derived therefrom, caused accelerated death. The substrate did not have to be utilized to cause death, because the gratuitous lac operon inducers isopropylthiogalactoside and methylthiogalactoside accelerated death of lactose-limited organisms. The suggestion of Postgate and Hunter (235), that the transfer of genetic information might be involved in substrate-accelerated death, was strengthened by Calcott and Postgate (33), who found that the addition of cyclic AMP prevented substrate-accelerated death in glycerol- and in lactose-limited populations. Measurement of intracellular cyclic AMP levels during starvation of lactose-limited cells in saline-phosphate buffer showed losses of 56% and 88% in the absence and presence of lactose respectively, during starvation (32). The corresponding death-rates under these conditions were

3% and 30% per h, respectively. This again demonstrated the acceleration of death by lactose and a corresponding loss of intracellular cyclic AMP. Calcott and Postgate (34) showed that K. aerogenes starved in the presence of lactose for 3 h and recovered on lactose-supplemented medium lost over 90% of their viability, but the addition of 5 mM cyclic AMP to the recovery medium allowed only a 25% loss of viability, the same as that of cells starved without lactose. Thus, cyclic AMP in the recovery medium could completely overcome accelerated death caused by lactose.

In summary, it appears that Gram positive organisms utilize endogenous amino acids preferentially during starvation. Both Gram positive and Gram negative organisms undergo substrate-accelerated death, which in Gram negative bacteria is apparently related to repression of enzyme synthesis caused by the substrate, mediated by cyclic AMP.

2. Levels of intermediates and of ATP in growing and in non-growing cells.

Because of the wide range of environmental conditions in which microorganisms grow, it is of interest to determine how these conditions affect their overall metabolic economy. Effects not only of temperature, pH, etc., but more importantly of a diversity of growth substrates may be reflected in the levels of various metabolic intermediates and in the ratios of the adenine nucleotides (ATP, ADP, and AMP), which serve as the energy currency of metabolism.

Lowry et al. (179) examined the differences in metabolite levels during logarithmic growth of E. coli on several carbon and nitrogen sources, and the effects of rapid nutrient changes on levels of intermediates. In the reference culture of cells grown with glucose and

ammonium ion, the levels of the EM pathway intermediates G6P and FDP were 1.8 and 6.6 μmol/g dry wt, respectively; the other EM intermediates were all at less than 1.0 μmol/g. Logarithmic growth in the presence of succinate or acetate instead of glucose resulted in lower FDP and dihydroxyacetone phosphate (DHAP) levels and higher levels of PEP. Addition of glucose to cells growing on succinate or acetate (i.e., gluconeogenically) resulted in much higher levels of G6P, FDP, and DHAP, but less PEP. The addition of glucose to cells growing on acetate, glycerol, or succinate caused only a slight reduction in the utilization of these compounds, and gluconeogenesis still continued, indicating that the conversion of succinate to PEP and the fructosediphosphatase reaction were not completely inhibited. Although the energy charge (4) of the cells remained fairly constant under all growth conditions, the ATP: AMP ratio varied from 2.0 to as high as 17.4. None of the levels of intermediates corresponded with the growth rate of the organisms.

Moses and Sharp (204) also measured intermediate levels in E. coli growing with various carbon sources and found that levels of FDP were consistently lower in cells growing on acetate, lactate, or succinate, i.e., gluconeogenically, than when growing with glucose, gluconate, glycerol, or ribose. The levels of ATP and ADP were relatively constant during growth with various carbon sources, although ATP exceeded ADP by a factor of 2.5-3. The levels of NAD and NADH in a number of bacteria were measured by Wimpenny and Firth (320), who found NAD consistently 2-4 fold higher than NADH. NAD in S. epidermidis grown aerobically in tryptone-glucose medium was present at 5.6 μmol/g vs 3.0 under anaerobic conditions. Oxygen did not affect NADH, which was present at 1.5 μmol/g aerobically and 1.6 μmol/g anaerobically. These values for S. epidermidis

were about the average for the five organisms tested.

Gancedo and Gancedo (91) surveyed the literature on the levels of metabolites in yeast under a variety of conditions, both growing and non-growing. All EM pathway intermediates, except FDP and 2- and 3-phosphoglycerate, were present at less than 1 mM. FDP ranged from 2.5 to 4.5 mM in cells growing on glucose or galactose. The concentrations of the adenine nucleotides were in the order ATP>ADP>AMP, with the concentration of ATP ranging from 1-5 mM in both growing and non-growing cells. NAD and NADH at average values of about 1.2 and 1 mM, respectively, were higher than in bacteria.

The mM concentration can be derived from μmol/g dry wt by multiplying by 0.2 g dry/g wet x 1.1 g wet/ml. This is based on the assumption that the dry weight of a bacterial cell is equal to about 20% of the wet weight, and that the density is 1.1 g/cc (204).

Because of its potential regulatory role, much interest has been shown in ATP and how its concentration is affected by various growth conditions. Forrest (78) found that the ATP pool size (the amount of ATP per unit volume of bacterial suspension) of anaerobically-growing Streptococcus faecalis was related to the growth rate, although the pool level (ATP per unit mass of bacteria) tended to decrease during the growth cycle. Forrest (78) suggested that the ATP level had only an indirect effect on the rate of growth of S. faecalis and that exponential growth requires an ATP pool above a critical level. Franzen and Binkley (86) used media that allowed from 0.73 to 2.40 doublings of E. coli/h and found that the levels of ATP, ADP, and AMP were essentially constant, averaging 3.9, 0.6, and 0.1 μmol/g, respectively.

Holms et al. (123) measured ATP in E. coli growing aerobically in a simple defined medium of salts, ammonium ion as the nitrogen source, and several different carbon sources. The ATP pool size (expressed as pmol/ml culture) increased linearly with the cell density during growth on all carbon sources (glucose, gluconate, acetate, glycerol, and malate). However, the ATP pool (μmol/g dry wt) and the growth rates with glucose, gluconate, glycerol, and malate were less variable. Growth of E. coli on limiting glucose or limiting glycerol indicated that the ATP pool (pmol/ml) generally paralleled the increase in cell density until stationary phase, when it decreased. Addition of glycerol after glycerol-limited growth had stopped caused an abrupt rise in the ATP pool (123). Lazdunski and Belaich (167) found that the ATP pool (μg ATP/mg dry wt) was constant during the log phase of Zymomonas mobilis during growth in minimal, defined, and complex media. At the beginning of glucose-limited growth, ATP underwent an increase from about 0.5 to almost 3.0 μg/mg, and then decreased to a constant level of 1.5 μg/mg, where it remained until growth ceased, when it fell to about 0.1 μg/mg.

Thus, in growing cells, the ATP pool size (ATP/ml culture) apparently increases, as might be expected, but the ATP per unit wt of cells remains constant (123,167) or tends to decrease (78). The constant ATP level during logarithmic growth suggests rapid turnover of the ATP pool and regulation of the reactions both producing and consuming it. The turnover rate of the ATP pool during growth with five different carbon sources was calculated by Holms et al. (123) to range from about 250 to 450 times per min.

The situation regarding ATP in non-growing cells differs considerably from that in growing cells. Forrest and Walker (79) grew Streptococcus

faecalis in a medium containing 2% glucose so that the cells were able to build endogenous reserves. Incubation of such cells in phosphate buffer (non-growing conditions) resulted in maintenance of a constant ATP pool for about 3 h. In contrast, the ATP pool in similar cells incubated in citrate buffer decreased by 47% in less than 1 h. A comparable loss of ATP occurred in cells that had no endogenous reserves and that had been incubated in phosphate buffer. Forrest (78,79) also observed that the ATP pool in starved cells incubated in phosphate buffer plus glucose increased by 7- to 10-fold under such non-growing conditions. Apparently, glucose catabolism yielded energy for ATP formation, which could not be used for growth.

Strange et al. (281) starved Enterobacter (Aerobacter) aerogenes aerobically in phosphate buffer and found reduced levels of ATP. However, transfer of cells from an anaerobic to an aerobic environment caused an abrupt 4-fold increase in the ATP level. Cole et al. (41) also observed an immediate rise in the level of ATP in E. coli when glucose was added to cells starved in buffer. A constant ATP pool in washed E. coli was observed for several hours by Wimpenny (319), who also found that addition of glucose caused a rapid increase in the pool size.

In summary, non-growing cells still carry on glycolysis at a constant rate that is relatively unaffected by additions of phosphate or amino acids (79). The ATP pool remains high and constant during starvation in phosphate buffer, falls rapidly in the absence of phosphate, but rises sharply when an energy source, such as glucose, is added.

C. In vitro properties of isolated enzymes

1. Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49)

Some of the properties of glucose-6-phosphate dehydrogenase (G6PD) in relation to regulation of the HMP pathway were considered in a previous section. Olive (218) divided G6PD into three groups on the basis of their coenzyme specificity. One group reacts only with $NADP^+$, and includes the enzymes from E. coli (254), Candida utilis (2), and Neurospora (259). A second group reacts with $NADP^+$ but also weakly with NAD^+ and includes the animal enzymes (172). The third group reacts equally with $NADP^+$ and NAD^+ and includes the enzymes from Pseudomonas aeruginosa (171), Leuconostoc mesenteroides (53), and Hydrogenomonas eutropha (23). The effectors of G6PD include ATP, AMP, NADPH, and NADH, which inhibit the enzyme from a variety of sources (318). Recently, Montiel et al. (200) reported the discovery in S. aureus of isozymes of G6PD, one of which was specific for $NADP^+$, and a second that could react with either $NADP^+$ or NAD^+ if the latter were at a concentration 5-10 times higher than that of $NADP^+$. The relatively low specific activity of G6PD found by Bluhm and Ordal (15) suggested that this enzyme might be rate-limiting in the HMP pathway in S. aureus. The literature on G6PD has been reviewed (22,230).

2. 6-phosphogluconate dehydrogenase (E.C.1.1.1.43)

Some of the early work on 6-phosphogluconate dehydrogenase (6PGD) was reviewed by Noltmann and Kuby (215). The enzymes from E. coli, yeast, and liver appeared to be specific for $NADP^+$, but the 6PGD from Leuconostoc reacted with both $NADP^+$ and NAD^+. Westwood and Doelle (318) tested various effectors on the enzyme from a number of organisms and all were inhibited by ATP, FDP, or NADPH, except those from Streptococcus faecalis and human erythrocytes, which were unaffected by ATP, and 6PGD

from Leuconostoc mesenteroides, which was unaffected by FDP. Brown and Wittenberger (26) found that growth in the presence of gluconate induced a NAD^+-specific 6PGD in Streptococcus faecalis. This enzyme was inhibited by ATP but not by FDP, whereas the $NADP^+$-linked 6PGD was inhibited by FDP and unaffected by ATP. The presence of a free sulfhydryl group may be a general requirement for activity of the microbial 6PGD. The enzymes from E. coli (318), Bacillus stearothermophilus (304), and Neurospora (258) were inhibited by p-hydroxymercuribenzoate, iodoacetate, or iodoacetamide, which are alkylating reagents that attack sulfhydryl groups. The enzymes from B. stearothermophilus and Neurospora were, however, completely and partially, respectively, protected from the SH reagent by the substrate, 6PG. The inhibition of 6PGD by FDP is also a common characteristic (318).

3. Fructosediphosphate aldolase (E.C.4.1.2.13)

This enzyme has been found in almost every organism examined, one exception being Leuconostoc mesenteroides, in which the EM pathway is absent (130). A division of aldolases into two major classes was proposed by Rutter (103,250). Class I aldolases occur in animals and in higher plants, including green algae. Their basic mechanism involves the formation of a Schiff base between an amino group in the enzyme and the keto group of DHAP (201). The class I aldolases do not require a metal ion cofactor, hence they are not inhibited by the chelating agent ethylenediamine tetracetic acid (EDTA), although the enzyme is inactivated by sodium borohydride in the presence of the substrate. Class II aldolases occur in bacteria, fungi, and blue-green algae, and are about half the molecular weight of the class I enzymes, generally 40,000-

70,000. The class II aldolases require a metal ion cofactor, most commonly zinc, hence they are sensitive to EDTA. Sodium borohydride does not affect class II aldolases, also called metalloaldolases.

An exception to the division of class I and class II aldolases between eukaryotic and prokaryotic organisms was noted by Lebherz and Rutter (169), who found that Micrococcus pyogenes, an anaerobe, contained a class I aldolase, which had the unique feature of being composed of a single polypeptide of molecular weight 33,000. Kaklij and Nadkarni reported discovery of class I aldolases in Lactobacillus casei, which were similar to rabbit muscle aldolase (145) and occurred in multiple forms (146). More significant perhaps was the realization that the three forms of aldolase had different activities both with respect to substrate and carbon source for growth of the organisms (144). Both classes of aldolase have been found in Euglena and Chlamydomonas (250), E. coli (284), and Lactobacillus casei (174).

Aldolases are generally not considered as regulatory enzymes. It appears that organisms synthesize aldolases that are kinetically appropriate to their particular situation. This applies to mammals, with organ-specific aldolases (130,169), as well as to microorganisms, in which aldolase synthesis is affected by the carbon source (144,284) or by the oxygen tension of the medium, as shown for E. coli (293). An exception to this was the report by Mattoo and Rao (188), who found that pyruvate inhibited FDP aldolase from Neurospora.

4. Glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12)

This is one of the key enzymes in carbohydrate metabolism, as it functions directly in both EM and HMP pathways. It is the first

enzyme in the constant proportion group (227). GAPD is ubiquitous, but most of the work has been done on the enzymes from rabbit muscle and yeast (302). Some of the features of rabbit muscle GAPD, isolated by Cori et al. (45), included a requirement of a reducing agent (cysteine or glutathione) for maximum activity (46) and a firmly-bound NAD^+ coenzyme that was not removable by dialysis (290). This enzyme is also particularly sensitive to irreversible inhibition by the alkylating agent iodoacetic acid (44), and to inhibition by p-chloromercuribenzoate, which is reversible by cysteine (300). Such effects suggested the involvement of a cysteine residue at the active site, and Harris (108) isolated a labeled cysteine derivative after treatment of the rabbit muscle enzyme with ^{14}C-iodoacetate. Replacing phosphate with arsenate allows the GAPD reaction to go to completion (303,312), spontaneously forming 3-phosphoglyceric acid. Arsenate is now used routinely in assays of GAPD.

The normal coenzyme for GAPD in animal tissues is NAD^+, but Brown and Wittenberger (25) described two forms of GAPD in Streptococcus mutans, utilizing NAD^+ or $NADP^+$ as coenzyme.

5. Pyruvate kinase (E.C.2.7.1.40)

The reactions PEP⇌PYRUVATE and FRUCTOSE-6-P⇌FDP are generally considered to be the key regulatory steps in the EM pathway because they are essentially irreversible in the glycolytic direction (262). Therefore, separate sets of enzymes and control mechanisms evolved to avoid the waste of energy that would occur caused by such uncontrolled "futile cycles" (260).

The most important regulatory feature of pyruvate kinase (PK) is

activation by FDP, first demonstrated for the yeast enzyme by Hess et al. (119). Such "feed-forward" activation has since been shown to occur with the enzymes from E. coli (180), Neurospora crassa (299), and other organisms (149). However, PK from a number of bacteria is apparently not activated by FDP (149).

Malcovati and Kornberg (159,184,185) reported the separation of two forms of pyruvate kinase in E. coli, only one of which was activated by FDP. Gibriel and Doelle (95) demonstrated that E. coli synthesized different forms of PK under aerobic and anaerobic conditions.

6. Fructosediphosphatase (E.C.3.1.3.11)

The first five enzymes discussed are part of the glycolytic EM pathway. Fructosediphosphatase (FDPase) is involved when organisms are growing on acetate, succinate, or amino acids.

FDPase was first discovered in mammalian tissues by Gomori (98). The first bacterial FDPase was found by Fossitt and Bernstein (81) in Pseudomonas saccharophila. It has since been found in E. coli (83), Bacillus licheniformis (220), and a wide variety of organisms (231).

To emphasize the amphibolic (50) nature of the EM pathway, the recent discovery of the activation of FDPase by PEP seems appropriate (8,330).

Each of the enzymes discussed here, except FDPase (which was not assayed), were found in S. aureus extracts by Bluhm and Ordal (15).

MATERIALS AND METHODS

A. Organisms and culture conditions

1. Organisms

The primary organism used in this investigation was Staphylococcus aureus Towler, a hospital strain isolated in England in 1963 by Dr. H. J. Blumenthal. For comparison, other staphylococcal strains used were S. aureus Peoria and S. aureus B VIII (coagulase-positive), S. epidermidis 7292 (prototype strain), S. epidermidis UA724 (pathogenic), and S. aureus BMcK (a coagulase-negative mutant of S. aureus B VIII). These strains were provided by Dr. H. Farkas-Himsley.

Three other bacteria were obtained from departmental sources. These were Bacillus cereus T (from Dr. T. Hashimoto), Escherichia coli (clinical laboratory strain), and Pseudomonas aeruginosa (clinical isolate supplied by P. van Gorder).

2. Maintenance of staphylococcal strains

S. aureus Towler was maintained on Trypticase Soy agar (TSA) slants, 40 g/l (BBL #11043, Cockeysville, MD). After each monthly transfer, organisms were incubated at 37 C for about 24 h, then stored at 4 C. The other staphylococcal strains were treated similarly.

3. Growth media

For the majority of experiments, S. aureus (the Towler strain) was grown in 2% (w/v) Vitamin-Free Casitone (VFC) (Difco #0646-01, Detroit, MI) or 2% Vitamin-Free Casamino Acids (VFCA) (Difco #0288-01), supplemented with 2 µg each of niacin (nicotinic acid) and thiamine-HCl per ml. Other media used were unsupplemented Trypticase Soy broth (TSB),

30 g/l (BBL #11768); and N-Z-Amine AS (a pancreatic digest of casein) and Edamin (a lactalbumin hydrolysate), provided by Humko-Sheffield (Lyndhurst, NJ). The latter two media were used at 2% (w/v) concentration, supplemented with 2 μg each of niacin and thiamine per ml.

4. Preparation of growth media.

The dry powder was added to either 500 ml or to 1 l of deionized and distilled water in 2 l flasks, dissolved, and dispensed to usually two to four 2 l flasks, depending on the quantity of cells required. After the medium was autoclaved at 121 C for 15 min, it was allowed to cool for several h before the membrane filter-sterilized vitamins were added.

The stock vitamin solutions were niacin (Nutritional Biochemicals, Cleveland, OH), 200 μg/ml, and thiamine-HCl (J.T. Baker, Phillipsburg, NJ), 400 μg/ml. Solutions were prepared by dissolving 40 mg of niacin in 200 ml of deionized and distilled water or 40 mg of thiamine in 100 ml of water in volumetric flasks. Solutions were prepared every few weeks and stored at -20 C.

5. Inoculation of growth media.

The standard procedure used for each experiment was as follows: A quantity of cells from the stock slant culture of S. aureus was streaked onto a TSA plate and incubated at 37 C overnight. A loopful of growth was inoculated into a (250 ml Erlenmeyer) starter flask containing 100 ml of unsupplemented 2% VFC or VFCA and incubated for 6-7 h at 37 C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 200 rev/min.

Just prior to inoculation of the growth medium, vitamin solutions were filter-sterilized and added to the growth medium. The filters were Millipore 13 mm diam., 0.45 μm pore size, contained in a plastic Swinnex holder (Millipore Corp., Bedford, MA), which was wrapped in brown paper (8x8 cm) and autoclaved at 121 C for 15 min. The filters were soaked briefly in deionized and distilled water prior to assembly to prevent drying and possible cracking during autoclaving.

To sterilize the vitamin solutions, a filter assembly was unwrapped and attached to a washed, disposable 10 cc syringe (Becton, Dickinson, Rutherford, NJ). About 12 ml of vitamin solution was poured into the barrel, the plunger was replaced, and the solution was filtered into a sterilized (screw-cap) glass tube. The filtration procedure was carried out reasonably close to a flame.

A concentration of 2 μg of vitamins/ml in 500 ml of medium required 1000 μg of vitamins to be added. Therefore, 5.0 ml niacin stock solution and 2.5 ml thiamine stock solution were added to each growth flask, using 5 ml cotton-plugged, sterile glass pipets. Immediately thereafter, a 1% inoculum (5 ml per 500 ml growth medium) of starter culture was added to each 2 l growth flask, using sterile 5-ml cotton-plugged glass pipets. The growth flasks, with cheesecloth-wrapped cotton closures covered with brown paper, were then incubated for 17 h at 37 C and 200 rev/min.

The turbidity of the starter culture was 20-40 Klett units. The turbidity of the 17-h cultures varied with the medium, and ranged from 150-280 Klett units. This was measured using a Klett-Summerson photoelectric colorimeter (Model 800-3, Klett Mfg. Co., New York, NY), equipped with a red (660 nm) filter, using uninoculated medium as blank.

VFC and VFCA were initially pH 5.5-6.0, changing to pH 7.1-7.2 after growth of S. aureus.

6. Harvesting of cells

After 17 h of growth (late log-early stationary phase), S. aureus was harvested by centrifugation in 250-ml plastic centrifuge bottles at 7,500 x g and 4 C for 10 min (Model B-20 centrifuge, International Equipment Co., Needham Hts., MA). Bottles were filled approximately two-thirds full and balanced with 0.05 M potassium phosphate (K-Pi) buffer, pH 7.0. A set of 6 centrifuge bottles was sufficient to harvest 1 l of culture medium. After decanting the supernatant fluid, more culture fluid was added to the centrifuge bottles, if necessary, to harvest the remaining cells. After decanting the supernatant fluid, about 10 ml of 0.05 M K-Pi buffer (pH 7.0) was added to each bottle, and the pellets were resuspended either by rapid intake and expulsion of liquid from a 5-ml glass pipet or by agitation on a Vortex mixer. The resulting cell suspensions were gathered into one centrifuge bottle, to which was added 10-20 ml K-Pi buffer rinse from the 5 other bottles. Thus, each S. aureus culture was washed once in about 80 ml 0.05 M K-Pi buffer, pH 7.0. After centrifugation to wash the cells, the pellet was resuspended in a volume of buffer determined, in part, by the experiment. Generally, the cells were suspended in 5 ml 0.05 M K-Pi buffer, pH 7.0, for each incubation flask and 5 ml for the initial (zero time) phenol extraction, plus an additional 3-5 ml if pathways were to be estimated at zero time.

B. Incubation and phenol extraction of cells

1. Incubation of cells

S. aureus was incubated under aerobic, non-growing conditions in 100 ml of medium in 250 ml Erlenmeyer flasks, or in 300 ml medium in 2 l flasks, as indicated in Results. The incubation medium generally consisted of 95 ml 0.05 M K-Pi buffer, pH 7.0, and 1.0 g D-glucose (PBG), plus other additions as noted. To this was added 5 ml of the washed cell suspension, to yield a total of 100 ml and 1% final glucose concentration. This particular medium served as the control, which was present in every experiment. Incubations were at 37 C and 200 rev/min, for 2, 3, or 6 h.

Additions to the incubation flasks included carbohydrates other than glucose, other buffers, and amino acids. When amino acids were added, the medium was adjusted to pH 7.0 when necessary, with either 2 M HCl or 2 M KOH and measuring with either a pH meter (Sargent-Welch model LS with a Corning 476051 glass combination electrode) or pH paper (pHydrion, ranges 1-14 and 6-8, Micro Essential Lab., Brooklyn, NY), prior to addition of cells.

After incubation, the cells were harvested in 250 ml centrifuge bottles or in 50 ml centrifuge tubes at 7,000 x g and 4 C for 10 min. The cells were washed once in 0.05 M K-Pi buffer, pH 7.0, by resuspending the cells in 10 ml buffer, transferring to 30-ml glass Corex centrifuge tubes (Corning Glass Works, Corning, NY), rinsing the centrifuge bottles with 5 ml buffer and transferring to the Corex tubes , and centrifuging. Depending on the experiment, the pellet was either resuspended in 10-12 ml buffer for the pathways estimation or extracted with phenol.

2. Phenol extraction of S. aureus

The method is that of Neuhoff (211). After cells were harvested,

either at zero time from the growth medium or after incubation, they were washed once by suspending in 15 ml 0.05 M K-Pi buffer, transferring the suspension to tared 30-ml Corex centrifuge tubes, and centrifuging. The Corex tubes were inverted and allowed to drain and were then carefully wiped free of excess liquid with Kimwipes (Kimberly-Clark Corp., Neenah, WI). The wet wt of cells was measured using a Mettler model H10 balance (Mettler Instrument Corp., Hightstown, NJ). The pellet was suspended in extraction buffer, consisting of 0.04 M tris (hydroxymethylamino) methane (Tris) base, 3.5×10^{-3} M ethylene-diaminetetraacetate (EDTA), disodium salt, and 5.0×10^{-3} M cysteine-HCl, final pH 7.5. The volume of buffer added was equal to ten times the wet wt of cells. The pellet was resuspended by agitation on a Vortex Jr. mixer (Scientific Industries, Inc., Springfield, MA), usually with a microspatula in the liquid to aid suspension. A volume of 89% phenol (Mallinckrodt "Gilt Label," U.S.P.), equal to 10% of the extraction buffer, was then added, dropwise over a period of 10-15 sec with vortex mixing to ensure even distribution of phenol in the cell suspension. The volumes of extraction buffer and phenol added were generally 3-7 ml and 0.3-0.7 ml, respectively. During addition of phenol, the consistency of the cell suspension increased from smooth and watery to a thicker mixture containing small clumps of precipitated cells. The color of the suspension also usually changed from a light golden or white to a light pink. After thorough mixing of phenol in the cell suspension, 10 ml of chloroform was added by gravity feed to the suspension with vortex mixing. After adding an additional 10 ml of chloroform (without vortex mixing) and balancing the Corex tubes against each other with chloroform, the tubes were centrifuged at about 13,000 x g and 4 C for 10 min. The resulting layers were an

upper aqueous phase containing the extracted cell sap, a thin middle layer of cell debris, and a bottom layer of chloroform and phenol.

The aqueous layer was then carefully transferred, using a 5 3/4" disposable (Pasteur) pipet, to a second Corex tube, followed by rapid addition of 20 ml of chloroform in 2 10-ml portions, and centrifugation. The upper layer was transferred to test tubes (either 16 x 125 mm or 12 x 100 mm), which were placed into a 60 C water bath to drive off any remaining chloroform. During the 3-min heating period, the tubes were swirled 4-5 times to aid removal of chloroform. The volume of each extract was measured in 10-ml glass graduated cylinders, after which the extracts were poured into 12 x 100 mm serological test tubes, which were immediately stored at -20 C, prior to assay of intermediates.

3. Assays of metabolic intermediates

The methods used were essentially those of Bergmeyer (10) or of Lowry et al. (179) with some modifications. Intermediates were assayed spectrophotometrically with a Gilford model 2000 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with a Beckman model DUR optical system (Beckman Instruments, Inc., Fullerton, CA). The wavelength was 340 nm, supplied by a tungsten lamp and a (blue) stray light filter. Assays were done at room temperature (about 24 C), in quartz cuvettes (capacity 1.75 or 4.0 ml) with a 1 cm light path.

All assays were done enzymatically, using reactions coupled directly or indirectly to reduction of nicotinamide adenine dinucleotide (NAD^+) or NAD^+ phosphate ($NADP^+$), or to the oxidation of NADH. For simplicity and clarity, the assay procedures are presented in Table 1.

In general before analysis, the frozen phenol extracts were

Table 1

The amount of each reagent, in μmol, is indicated, except for enzymes, which are expressed as units (U).

1. FDP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate: GAP, glyceraldehyde-3-phosphate. GDH, α-glycerolphosphate dehydrogenase; L-glycerol-3-phosphate: NAD oxidoreductase, E.C. No.1.1.1.8. Sigma type 1, from rabbit muscle. Measures DHAP. TPI, triosephosphate isomerase, E.C. No.5.3.1.1. Sigma type III, from rabbit muscle. Measures GAP. ALD, FDP aldolase; fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, E.C. No.4.1.2.7. Sigma grade 1, from rabbit muscle. Measures FDP.

The FDP assay involved the oxidation of NADH. The initial readings were generally 1.2-1.5 A, well within the range of the spectrophotometer. With high concentrations of FDP, the decrease in A was as much as 1.0 A.

2. ATP, adenosine-5'-triphosphate; G6P, glucose-6-phosphate. TEA, triethanolamine buffer. G6PD, glucose-6-phosphate dehydrogenase. Sigma type XI, from Torula yeast. Hexokinase, Sigma type III, from yeast.

3. F6P, fructose-6-phosphate. PGI, phosphoglucose isomerase; D-glucose-6-phosphate ketol isomerase. E.C. No.5.3.1.9. Sigma grade III, from yeast.

4. 6PGD, 6-phosphogluconate dehydrogenase; 6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating). E.C. No.1.1.1.44. Sigma type IV, from yeast.

5. PEP, phosphoenolpyruvate. LDH, lactic dehydrogenase; L-lactate: NAD oxidoreductase. E.C. No.1.1.1.27. Sigma type III, from beef heart. PK, pyruvate kinase; ATP: pyruvate phosphotransferase. E.C. No.2.7.1.40. Sigma type II, from rabbit skeletal muscle.

6. NAD, nicotinamide adenine dinucleotide. (Does not include NADH.) ADH alcohol dehydrogenase. Sigma, from yeast.

7. NADP, NAD phosphate.

Table 1. Procedures for assay of metabolic intermediates

1. FDP-DHAP-GAP

	ml	µmol	
(1) 0.1 M Tris pH 8.8	2.00	200	
(2) Extract	1.00 max	--	
(3) 0.035 M NADH	0.02	0.7	A1
(4) GDH	0.02	3U	A2
(5) TPI	0.02	8U	A3
(6) ALD	0.02	0.3U	A4
	3.08		

2. ATP-G6P

	ml	µmol	
(1) 0.2 M TEA pH 7.5	1.00	200	
(2) 0.04 M NADP	0.02	0.8	
(3) 0.1 M $MgCl_2$	0.02	2	
(4) Extract	0.50	--	
(5) H_2O	0.50	--	A1
(6) G6PD	0.02	1U	A2
(7) 0.5 M Glucose	0.25	125	A3
(8) Hexokinase	0.02	3U	A4
	2.33		

3. G6P-F6P

	ml	µmol	
(1) 0.2 M Tris pH 7.5	0.50	100	
(2) 0.5 M $MgCl_2$	0.01	5	
(3) 0.08 M NADP	0.01	0.8	
(4) Extract	0.50	--	A1
(5) G6PD	0.01	3U	A2
(6) PGI	0.01	0.6U	A3
	1.04		

4. 6PG-G6P

	ml	µmol	
(1) 0.2 M Tris pH 7.5	0.50	100	
(2) 0.025 M NADP	0.02	0.5	
(3) 0.5 M $MgCl_2$	0.01	5	
(4) Extract	0.50	--	A1
(5) 6PGD	0.01	1U	A2
(6) G6PD	0.02	1U	A3
	1.06		

Table 1, continued

5. PEP-PYRUVATE

	ml	µmol	
(1) 0.2 M Tris pH 7.5	0.50	100	
(2) 2 M KCl	0.02	40	
(3) 0.5 M $MgCl_2$	0.01	5	
(4) 0.01 M ADP	0.01	0.1	
(5) 0.02 M NADH	0.01	0.2	
(6) Extract	0.50	--	A1
(7) LDH	0.01	8U	A2
(8) PK	0.01	4U	A3
	1.07		

6. NAD

	ml	µmol	
(1) 0.2 M Tris pH 8.5	0.50	100	
(2) Extract	0.50	--	
(3) Absolute ethanol	0.02	343	A1
(4) ADH	0.02	3U	A2
	1.04		

7. NADP

	ml	µmol	
(1) 0.2 M Tris pH 8.5	0.50	100	
(2) Extract	0.50	--	
(3) 1 M $MgCl_2$	0.01	10	
(4) 0.1 M G6P	0.02	2	A1
(5) G6PD	0.01	3U	A2
	1.04		

allowed to thaw at room temperature, and were then mixed thoroughly, either on a vortex mixer or by covering the opening with Parafilm (American Can Co., Neenah, WI) and inverting the tube 5-6 times. The tubes were centrifuged (IEC International Clinical Centrifuge model CL, Needham Hts., MA; position 6) for 10 min to sediment precipitated material.

After the additions indicated in Table 1 and before reading, the cuvette opening was covered with Parafilm and the cuvette was inverted 3-4 times to thoroughly mix the contents. A buffer blank was included in each set of cuvettes. Absorbance readings were made directly on the instrument and were taken at intervals of 1-5 min, depending on the reaction and its rate.

Most of the reactions were rapid initially and then gradually decelerated and stopped, i.e., no further absorbance change occurred. Exceptions to this occurred during assays of intermediates at low concentration, such as analysis of most zero time samples. Another exception involved the ATP-G6P and PEP-pyruvate assays, where the reactions of ATP and PEP were initially rapid, then decelerated, and did not actually stop abruptly, but rather continued at a slow, constant rate (absorbance change of 0.004-0.007/min) for as long as 10 min. Therefore, the absorbance change endpoint was interpolated by subtracting the slow, constant change from the more rapid and decelerating absorbance change until zero change was obtained. This was a satisfactory method because results were reproducible.

At the conclusion of an assay, a known amount of substrate was added directly to the cuvette in which the assay had been conducted and the additional absorbance change was noted. This accomplished the following: (1) An absorbance change (calculated from the Beer-Lambert Law)

proportional to the amount of substrate added indicated that the overall assay system functioned properly. (2) It indicated that the enzymes present were still functioning. (3) It indicated that the reaction stopped because the unknown amount of substrate was consumed and not because the coenzyme or some other component in the system was limiting. Addition of such known amounts of substrates indicated that the assay systems were reliable. Most reacted at least 80-90% of the expected amount, but FDP and NAD were the most reliable with an efficiency of 95-100%. Of course, these measurements also depended to a large extent on the accuracy with which the solutions of the known substrates were prepared.

The levels of intermediates reported are based on absorbance changes of 0.05 or more, as the results of changes less than 0.05 were considered unreliable and are reported as < 1 μmol/g. With the assay systems used, an absorbance change of 0.05 calculated to approximately 1 μmol/g dry wt. The corresponding concentration of substrate in the cuvette was about 0.01 μmol/ml.

4. Calculation of intracellular concentrations of intermediates

The concentrations of intermediates are expressed as μmol intermediate/g dry wt of cells. Since a bacterial cell is approximately 80% water (204), the wet weight was multiplied by 0.2 to obtain the dry weight. The equations used in the calculations were derived as follows:

The basic equation is that expressing the Beer-Lambert Law

$$A = abc \qquad (1)$$

where A = absorbance (formerly O.D., optical density; no units), a = molar absorptivity, in l/mole-cm, b = light path length in cm, and c = concentration in moles/liter. Concentration is the ultimately desired

expression, hence, rearranging equation (1) yields

$$c = A/ab \qquad (2)$$

The amount of extract added is only a fraction of the total volume in the cuvette. This dilution factor = (total volume in cuvette/volume of sample) and has no units. Therefore,

$$C = (\Delta A)\ (DF)/ab, \qquad (3)$$

where ΔA = the change in absorbance caused by the reaction in the cuvette. This equation would yield the concentration of substrate in the cuvette, so to determine the concentration in the phenol extract, which represents the total cell sample, the numerator is multiplied by the total volume of extract obtained, and the denominator is multiplied by the g dry wt of cells (the wet wt x 0.2):

$$C = \frac{(\Delta A)\ (DF)\ (\text{Vol of extract in l})}{(a)\ (b) \quad (\text{g dry wt})} \qquad (4)$$

To convert from mol to μmol, the numerator is multiplied by 10^6 μmol/mol. And because NAD is used as the chromophore, a = 6.22×10^3 (13). The complete equation is:

$$C = \frac{(\Delta A)\ (DF)\ (\text{vol. of extract in l})\ (10^6\ \mu\text{mol/mole})}{(6.22\text{x}10^3\ \text{l/mol-cm})\ (1\ \text{cm})\ (\text{g dry cells})} \qquad (5)$$

To simplify the calculation by cancelling exponents, the volume of extract is expressed as ml, which equals 1×10^{-3}. Therefore,

$$C = \frac{(\Delta A)\ (DF)\ (\text{vol. of extract in } 1 \times 10^{-3})\ (10^6\ \mu\text{mol/mol})}{(6.22 \times 10^3\ \text{l/mol-cm})\ (1\ \text{cm})\ (\quad \text{g dry wt})} \qquad (6)$$

Cancelling and collecting terms, the final equation, and that used in the calculations, is:

$$\mu\text{mol}_{/\text{g dry wt}} = \frac{(\Delta A)\ (DF)\ (\text{vol. of extract in ml})}{(6.22)\quad (\text{g dry wt})} \qquad (7)$$

5. Anaerobic growth and incubation of S. aureus

Only preliminary experiments were done with S. aureus under anaerobic and semi-anaerobic conditions. Two approaches were used, one for growing cells anaerobically and one for growing them aerobically and then incubating them under semi-anaerobic or anaerobic conditions.

Cells were first incubated aerobically for 6 h in a starter flask containing 100 ml 2% VFC. The growth medium was 2% VFC supplemented with 0.02 M Na pyruvate and 1.0 mM uracil contained in 250 ml plastic (Nalgene polycarbonate) screw cap flasks, filled almost to the top, autoclaved, and cooled (155). A marble (vol. about 2 ml) was also present in each flask to provide some mixing of the contents during shaking (137). The autoclaved medium was used soon after preparation to retard oxygen absorption into the medium from the air. Flasks were inoculated with 1 ml of niacin and thiamine solution (final concentration of each, 2 μg/ml) and 2.5 ml of starter culture, then filled to the top with additional medium and closed with the screw caps. The flasks were incubated with shaking at 37 C for 17 h (final cell density averaged 43 Klett units), the cells were harvested, and incubated aerobically in 0.1 M K-Pi buffer, pH 7.0, containing 1% glucose.

The second approach involved anaerobic incubation of aerobically-grown S. aureus suspensions. Cells were grown aerobically in 2% VFC or in TSB as described previously, harvested, washed once in 0.05 M K-Pi buffer, pH 7.0, and resuspended in 0.05 M K-Pi buffer, pH 7.0. After adding 5-ml aliquots of cells to 95 ml of incubation medium contained in 250-ml Erlenmeyer flasks, the cells were incubated for 3 h aerobically, as described previously, and under three other conditions. The

first was semi-aerobic and involved minimal stirring of the incubation medium with a 1.5 in Teflon-coated stirring bar spinning slowly in the medium, with rotation provided by a Tek Stir stirring apparatus (Scientific Products, McGaw Park, IL). The second was also semi-aerobic and the flask was simply allowed to stand undisturbed at 37 C. The third method was a true anaerobic incubation. The flasks were placed into an anaerobic incubator (Precision Thelco, Precision Scientific Co., Chicago, IL), which was then evacuated by a vacuum pump to -25 in Hg for about 6 min, then filled with CO_2 to a positive pressure of 1.5 pounds. After 3 h of incubation under these conditions, cells were harvested and extracted with phenol as described previously.

C. Radiorespirometric methods

1. Estimation of glucose catabolic pathways by radiorespirometry

The percentages of glucose catabolized by the pathways were estimated by the method of Wang et al. (311) as modified by Blumenthal (16). After harvesting and washing, the cells were suspended to a density of 1200 Klett units in 0.05 M K-Pi buffer, pH 7.0, determined by adjusting a 1:10 dilution to read 120 Klett units. The cell density was determined in Klett tubes in a Klett-Summerson photoelectric colorimeter with a red (660 nm) filter and a buffer blank. The incubation mixture consisted of 1.0 ml 0.1 M K-Pi buffer, pH 7.0, 0.5 ml glucose-1-^{14}C or -6-^{14}C solution (50 μmol/ml; about 150,000 counts/min/ml or 0.1 μCi/ml), and 2.5 ml cell suspension (total vol. 4.0 ml). The estimations were done in 50 ml Erlenmeyer flasks with a center well and a 16 mm opening that could be closed with a rubber serological stopper. In the center well was placed a 10 x 22 mm

test tube containing Whatman No. 1 chromatography paper (87 g/m^2, 0.16 mm thick, medium flow rate), 30 x 35 mm, folded 4-5 times, which was saturated with 1 ml CO_2-absorbing fluid, consisting of a 1:2 mixture of ethanolamine and ethyleneglycol monomethylether.

Materials were added in the order: center well test tube (without fluid), buffer, glucose, cells, stopper. The cells were added to the estimation flasks at 1 min intervals, which allowed time to mix the flask contents, remove 0.5 ml of incubation mixture to the perchloric acid, stopper the flasks, and place them into the shaker. After the stopper was in place, the CO_2-absorbing fluid was added to the center well by injection through the rubber stopper using a disposable plastic 3 ml syringe with a 50 mm 21 gauge needle. The flasks, which were duplicates of both C-1 and C-6 glucose (i.e., 4 flasks/set) were placed into a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, IL) at 37 C and 50 cycles/min. Sufficient water was present in the shaker to exceed the level of fluid in the flasks by several mm.

At zero time (i.e., after the initial addition of cells) and after 2 h of incubation, 0.5 ml of incubation mixture was removed and added to 2.5 ml 10% perchloric acid contained in 12 x 100 mm test tubes. These samples were assayed for glucose to indicate the amount of glucose used by the cells during the 2 h incubation.

At the end of the 2 h incubation, the flasks were removed in the same order at 1 min intervals, the stoppers were removed, 0.5 ml of incubation mixture was added to the perchloric acid, and the center well test tubes were removed with a forceps and placed into glass scintillation vials (25 mm cap size, Packard Instrument Co., Inc., Downers Grove, IL).

To the scintillation vials were then added 10 ml scintillation fluid from a prepipetter attached to a 500 ml flask, plus 1 ml of the CO_2-absorbing fluid. The scintillation fluid consisted of 1.65 g 2,5-diphenyloxazole (PPO), 100 ml ethyleneglycol monomethylether, and 200 ml scintillation grade toluene.

The activity of the absorbed $^{14}CO_2$ was determined in an automatic liquid scintillation spectrometer (Packard Instrument Co., Tri-Carb model 3320). The activity of each vial was determined for 10 min, at which time the total counts were recorded. The calculations were based on the last two complete counts of the vials, after the vials were allowed to cool and equilibrate in the spectrometer for at least 6 h. A blank vial, consisting of the center well test tube and paper, was included and its counts were subtracted from each experimental vial. A ^{14}C standard vial of known activity (32, 787 dpm $\pm$ 1.3%) from the manufacturer, was also included in each run to insure uniform operation of the instrument. A uniform yield of about 23,000 cpm from the ^{14}C standard indicated that the counter operated with an efficiency of 70%. Two channels of the instrument were used for counting, with window settings of 50-1000 divisions and a gain setting of 14.25%. Each calculation was therefore based on 2 each of C-1 or C-6 flasks x 2 channels x 2 runs = 8 counts.

2. Assay of glucose

Glucose was assayed by the anthrone method of Seifter (261), slightly modified. Each test tube, containing 3 ml perchloric acid and cells, was mixed briefly on a vortex mixer and centrifuged for 5 min (Sorvall Angle Centrifuge, 90 volts). From the zero time and 2 h samples were removed 0.25 and 0.5 ml, respectively, which were placed into test tubes.

The volume of each was brought to 3.0 ml with water and the contents were mixed on a vortex mixer.

The anthrone reagent, a 0.2% solution in concentrated sulfuric acid, was prepared several h prior to the assay. Six ml of this solution were added by ejection from a 10 ml glass pipet to the 3 ml solutions contained in the test tubes. The test tubes were cooled in ice water to moderate the heating caused by the acid. Each tube was immediately vortex mixed to insure even distribution and reaction of the anthrone reagent and replaced in the ice bath. After all tubes were so treated, they were placed into a boiling water bath for 10 min to aid color development. The tubes were then cooled in the ice bath and read in a Klett colorimeter with a green (540 nm) filter. The instrument was set to zero using a water blank. A standard curve, using 30, 60, and 90 μg glucose, was prepared for each assay. The standard glucose concentration of 90 μg/ml gave a reading of generally 90-100 Klett units.

Because results were comparable to those using the more sensitive glucose oxidase procedure, the anthrone test was used routinely.

3. Calculation of pathway participation

Justification for the radiorespirometric method is based on the following considerations (310):

(i) Carbon 1 of glucose is removed as CO_2 during the 6-phosphogluconate dehydrogenase reaction. No additional CO_2 is evolved in the HMP pathway.

(ii) No CO_2 is given off in the EM pathway.

(iii) The two GAP molecules formed in the EM pathway are in equilibrium with each other and with respect to further reactions.

(iv) Carbons 3 and 4 of glucose are evolved as CO_2 during the pyruvate dehydrogenase reaction, converting pyruvate to acetyl coenzyme A.

(v) Neither the formation of hexoses from trioses nor the randomization of hexoses via the transketolase and transaldolase reactions in the HMP pathway occur to a significant extent.

(vi) Carbons 1 and 6, after randomization, eventually appear as CO_2 during the reactions of the TCA cycle.

(vii) The difference in radioactive counts between glucose-1-^{14}C and glucose-6-^{14}C represents metabolism via the HMP pathway exclusively.

(viii) The counts from glucose-6-^{14}C represent the portion of glucose oxidized via the complete TCA cycle.

Glucose catabolism via the HMP pathway was calculated using the equation

$$\% \text{ HMP} = \frac{C_1 - C_6}{C_T},$$

where C_1 and C_6 are the counts from glucose-1-^{14}C and glucose-6-^{14}C, respectively, and C_T is the total available counts, consisting of three factors: (i) the specific activity in cpm/ml of the stock labeled glucose solutions (or the higher specific activity if they differ); (ii) the constant 0.875, representing the fraction (7/8) of glucose remaining and available to the cells after removal of 0.5 ml of incubation mixture for the zero time glucose assay; and (iii) the percentage of glucose utilized by the cells, determined from the anthrone test.

If the specific activities of the stock labeled glucose solutions differed, the higher specific activity was divided by the lower to yield a correction factor. The counts from the glucose with the lower activity were multiplied by this factor to correct for the error that would have

been caused by the inherent difference in the labeled glucose solutions. The specific activity of the stock labeled glucose solutions was determined by adding 10, 20, and 30 µl of solution to scintillation vials in duplicate, adding 10 ml of scintillation fluid, and measuring the activity, which was corrected for background and multiplied by the appropriate dilution factor. The average of the counts from the 6 vials was used to calculate the specific activity.

The percent of glucose catabolized by the EM pathway was determined by subtracting the % HMP pathway from 100. Based on item (viii) above, an approximation of the activity of the TCA cycle was obtained from the expression C_6/C_T.

4. Calculation of the absolute amount of glucose utilized by the pathways

The total µmol of glucose utilized divided by 2 equaled the µmol of glucose utilized per h. This multiplied by the percentages of glucose used by the HMP and EM pathways yielded the absolute amounts of glucose utilized by the pathways.

5. Summary of the radiorespirometric method

The information necessary for estimation of the glucose catabolic pathways may be summarized in the following items:

From the scintillation counter:

(1) C_1 counts - blank,

(2) C_6 counts - blank,

(3) Equated specific activity,

(4) C_1 counts - C_6 counts.

From the anthrone test:

(5) Initial μmol glucose,

(6) Final μmol glucose,

(7) μmol glucose used,

(8) % glucose used,

(9) C_T = highest specific activity of glucose x 0.875 x % glucose used.

From the equation:

(10) % HMP = $(C_1-C_6)/C_T$,

(11) % EM = 100 - % HMP,

(12) TCA activity = C_6/C_T.

From the pathway percentages:

(13) μmol glucose used per h (from item 7),

(14) item (13) x % HMP = μmol glucose used via HMP pathway,

(15) item (13) x % EM = μmol glucose used via EM pathway.

D. Methods for assay of enzymes

1. Preparation of cell extracts

S. aureus Towler was grown in 2% VFC or VFCA supplemented with 2 μg each of niacin (nicotinic acid) and thiamine-HCl per ml, or in unsupplemented TSB (30 g/l) for 17 h. The cells were harvested by centrifugation in 250 ml plastic centrifuge bottles at 7,500 x g and 4 C for 10 min, then collected into one tared bottle or 50 ml centrifuge tube, and washed once or twice in 0.05 or 0.1 M Tris-HCl buffer, pH 7.5. After determining the wet wt of cells, they were suspended in a volume of 0.1 M Tris buffer plus glycerol equal to twice the wet wt. The proportions of buffer and glycerol were 80% and 20%, respectively. Glycerol was

included based on the finding of London that it helped stabilize FDP aldolase in cell-free extracts of Lactobacillus (175). The cell density of S. aureus in the buffer + glycerol averaged 0.46 g/ml (range 0.39-0.50). A volume of glass beads (25 μm diam; Heat Systems-Ultrasonics, Inc., Plainview, NY) equal to 1/4 the volume of the suspending fluid was also added. During sonication, the cell suspension was contained either in a plastic 50 ml centrifuge tube or in a "cold shoulders" cooling cell (Heat Systems - Ultrasonics, Inc.), which was partially immersed in an ethyleneglycol bath at a temperature of -5 to -9 C contained in a Forma-Temp Jr. refrigerated and heated bath and circulator (Forma Scientific Co., Marietta, OH).

The cell suspension was sonicated at maximum power for 1 min followed by 2 min of cooling for a total of 16 min of sonication using a Branson sonifier (model S75, Branson Instruments, Inc., Stamford, CT), equipped with a microtip. During the periods of cooling, the suspension was occasionally stirred with a plastic spatula. The maximum power obtained was 4-6 amp. During sonication, the temperature of the suspension increased to about 20 C.

After sonication was completed, the suspension, contained in a 50 ml plastic centrifuge tube, was centrifuged (IEC model B-20) at about 28,000 x g and 4 C for 30 min. The volume of supernatant fluid was measured in a chilled glass graduated cylinder and then poured into plastic bottles for storage at about -35 C. The pellet was resuspended in the same volume of buffer and glycerol and the sonication procedure was repeated. The resulting crude extracts were combined and used for assay of enzyme activity. Some of the crude extracts were fractionated with

ammonium sulfate.

2. Ammonium sulfate fractionation

After a short period of storage, a measured volume of crude cell extract was placed into a 100 ml beaker surrounded by ice in a pan atop a stirring apparatus. Enzyme grade ammonium sulfate (Mann Research Laboratories, New York, NY, now Schwarz/Mann, Orangeburg, NY) was ground to a powder with a mortar and pestle, and appropriate amounts were added slowly to the cell extract over a period of about 30 min with constant, slow stirring. During the addition of ammonium sulfate, the pH of the extract was monitored with pH paper and maintained at pH 7.0-7.5 with 1.5 M NH_4OH. After the last addition of ammonium sulfate, the extract was stirred for 5 additional min, then poured into a 50 ml plastic centrifuge tube, which was placed into ice and stored at 4 C for 1 h. The extract was then centrifuged at about 28,000 x g and -5 to 0 C for 30 min. The supernatant fluid was poured into a 100 ml beaker for the next fractionation step.

The ammonium sulfate fractions were prepared at 40%, 70%, and 100% saturation at 0 C. Amounts of ammonium sulfate to be added at 0 C were determined from a chart (52), but the following equation may also be used (289):

$$X = \frac{51.5\ (S2-S1)}{1-0.27\ S_2}$$

where X = g of ammonium sulfate to be added to 100 ml of extract, and S_1 and S_2 are the initial and final saturations, respectively.

After centrifugation, the pellet of each fraction was dissolved in a small volume of 0.05 M Tris buffer, pH 7.5. The fractions and the ammonium sulfate-saturated extract (above 100%) were stored at about -35 C.

3. Determination of protein concentration

The protein concentration in crude extracts and ammonium sulfate fractions was determined by the spectrophotometric method of Waddell (305). The absorbance of appropriate water dilutions of samples was measured at 215 and 225 nm, using quartz cuvettes, and a water blank. Crude extracts were diluted as much as 700-fold. The difference between these readings (215-225) x 144 x the dilution factor equalled the protein concentration in μg/ml. The protein concentration was the average of duplicate readings of at least three different dilutions. The method was found to be reliable by measuring dilutions of known concentrations of crystalline bovine serum albumin.

4. Assays of enzyme activity

The activities of several EM pathway enzymes were determined in crude extracts and ammonium sulfate fractions by reactions directly or indirectly coupled to NAD reduction or to NADH oxidation, with absorbance changes measured in a Gilford model 2000 recording spectrophotometer at 340 nm and maintained at 30 C using a circulating water bath.

The procedures for the assays (except for G6PD and 6PGD) were selected after surveying the literature (ca. 20-30 papers) for each enzyme. Many papers referred to "standard" assays and some of these are listed as references. However, the procedures used in this dissertation were composites or "averages" of the published assays, which were then tested and modified to yield the optimized standard assays used in this investigation and which are presented in Table 2.

In general, frozen extracts were allowed to thaw at room temperature and were then gently mixed before storage in an ice bath during the

Table 2

The asterisks indicate reagents used to initiate the reactions. The volume of extract is the maximum, and includes the extract and other additions. Where necessary, the balance of this volume was provided by water. The amounts of reagents are expressed as μmol and enzymes are expressed as units (U).

1. HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid. G6P, glucose-6-phosphate, disodium salt. NADP, monosodium salt.

2. 6PG, 6-phosphogluconic acid, trisodium salt.

3. FDP, fructose-1,6-diphosphate, tri-or tetrasodium salt. NaAs, sodium arsenate (Na_2HAsO_4), dibasic crystal. GAPD, glyceraldehyde-3-phosphate dehydrogenase, Sigma, from rabbit muscle. TPI, triosephosphate isomerase, Sigma type III, from rabbit muscle. NADH, reduced NAD, disodium salt. GDH, α-glycerophosphate dehydrogenase, Sigma type I, from rabbit muscle.

4. GAP, glyceraldehyde-3-phosphate, prepared and assayed as described in Materials and Methods. Cysteine, hydrochloride, neutralized to pH 6-7 with 2 M KOH.

5. PEP, phosphoenolpyruvic acid, trisodium salt. ADP, adenosine-5'-diphosphate, sodium salt. LDH, lactic dehydrogenase, Sigma type III, from beef heart.

6. G6PD, glucose-6-phosphate dehydrogenase, Sigma type XI, from Torula yeast. PGI, phosphoglucose isomerase, Sigma grade III, from yeast.

Table 2. Procedures for assay of enzyme activity

1. Glucose-6-Phosphate Dehydrogenase

	ml	µmol
(1) 0.1 M Hepes pH 7.3	1.00	100
(2) 0.03 M G6P*	0.04	1.2
(3) 0.01 M NADP	0.06	0.6
(4) Extract	0.10	--
	1.20	

2. 6-Phosphogluconate Dehydrogenase

	ml	µmol
(1) 0.1 M Hepes pH 7.3	1.00	100
(2) 0.03 M 6PG*	0.04	1.2
(3) 0.01 M NADP	0.06	0.6
(4) Extract	0.10	--
	1.20	

3a. FDP Aldolase, NAD Reduction

	ml	µmol
(1) 0.125 M Tris pH 7.5	0.40	50
(2) 0.05 M FDP	0.10	5
(3) 0.1 M NAD*	0.04	4
(4) 0.1 M NaAs	0.20	20
(5) GAPD	0.01	8U
(6) TPI	0.01	20U
(7) Extract	0.24	--
	1.00	

3a. Ref.: 20, 251

3b. FDP Aldolase, NADH Oxidation

	ml	µmol
(1) 0.1 M Tris pH 7.5	0.50	50
(2) 0.05 M FDP*	0.10	5
(3) 0.0025 M NADH	0.10	0.25
(4) GDH	0.01	16U
(5) TPI	0.02	20U
(6) Extract	0.27	--
	1.00	

Ref: 13, 101, 102, 328

Table 2, continued

4. Glyceraldehyde-3-Phosphate Dehydrogenase

	ml	µmol
(1) 0.125 M Tris pH 7.5	0.40	50
(2) GAP, 0.013 M avg.	0.08	1
(3) 0.1 M NAD*	0.01	1
(4) 0.1 M Na arsenate	0.10	10
(5) 0.05 M cysteine	0.10	5
(6) Extract	0.31	--
	1.00	

Ref.: 162, 301

5. Pyruvate Kinase

	ml	µmol
(1) 0.125 M Tris pH 7.5	0.40	50
(2) 0.04 M PEP*	0.05	2
(3) 0.02 M ADP	0.10	2
(4) 0.0025 M NADH	0.08	0.2
(5) 2 M KCl	0.04	80
(6) 0.2 M $MgCl_2$	0.05	10
(7) LDH	0.02	2U
(8) Extract	0.26	--
	1.00	

Ref.: 28, 138, 245

6. Fructose Diphosphatase

	ml	µmol
(1) 0.1 M Tris pH 7.5 or GLY pH 9.5	0.50	50
(2) 0.01 M FDP*	0.10	1
(3) 0.01 M NADP	0.05	0.5
(4) 0.2 M $MgCl_2$	0.10	20
(5) G6PD	0.02	1.2U
(6) PGI	0.02	4U
(7) Extract	0.21	--
	1.00	

Ref.: 84, 232, 287

assays. When activity was excessive, extracts were diluted up to 5-fold in 0.1 M Tris buffer, pH 7.5. All reagents except the substrate were added to the quartz cuvettes (1.75 ml capacity), which were then inverted several times to mix the contents. (The tops of the cuvettes were covered with Parafilm during mixing the prevent leakage.) The cuvettes were placed into the spectrophotometer, with the control in the first position, and the recorder scale was calibrated to an appropriate full scale range, generally from 0.8 - 1.2 A. The control cuvette was set at zero % on the scale (or near 100% for NADH oxidation), and the remaining cuvettes were set at 5%, 10%, and 15% (or proportional figures decreasing from the upper end of the scale) by means of an auxiliary offset control (Gilford Instrument Laboratories, Inc., model 208). The automatic cuvette positioner was engaged to read each cuvette with a 10 sec dwell. This continued for 3-5 min to allow the cuvettes to equilibrate to 30 C. The reaction was initiated by addition of substrate or coenzyme as indicated in Table 2, and the cuvettes were read automatically for 10 sec each for up to 10 min of reaction time. The resulting chart recording was used to calculate the activity of the enzymes.

5. Dialysis of crude extracts

Crude cell extract, a 70-100% fraction, and a supernatant fraction above 100% saturation were dialyzed to partially purify the extracts. A volume of extract was placed into Spectrapor membrane tubing (cylindrical diameter 15.9 mm, molecular weight cutoff 12,000-14,000; Spectrum Medical Industries, Inc., Los Angeles, CA) which was fitted with Spectrum plastic snap closures and placed into 500 ml of 0.05 M Tris buffer, pH 7.5, in a graduated cylinder atop a stirring

apparatus at about 4 C. The crude extracts were dialyzed once for 24 h and the ammonium sulfate fractions were each dialyzed twice for 3 h. At the end of dialysis, the final volume of extract was measured and the extracts were stored at -35 C. The ammonium ion present in the dialysate was monitored using ammonium test paper (Macherey, Nagel, and Co., Duren, West Germany) and was negligible at the end of the second dialysis.

6. Preparation and assay of glyceraldehyde-3-phosphate

The glyceraldehyde-3-phosphate (GAP) was prepared from the diethylacetal monobarium salt by ion exchange according to directions from Sigma Chemical Co., St. Louis, MO. In a test tube were suspended 1.5 g Dowex-50W H form (Sigma No. 50X4-200R), a strongly acid cation exchange resin, in 6 ml H_2O. To this was added 100 mg DL-GAP diethylacetal monobarium salt. The tube was placed into a boiling water bath for 3 min and was shaken intermittently, after which it was cooled in an ice bath. The tube was centrifuged (Sorvall Angle Centrifuge, about 100 volts) for 10 min, the supernatant fluid was decanted, and the resin was washed twice in 2 ml of water. The resulting solutions were combined, adjusted with 2 M KOH from an initial pH of about 2 to pH 6-7, and assayed. Solutions were stored at -30 C.

The assay of GAP was modified from that of Lowry et al. (179) and consisted of 2.0 ml 0.1 M glycine buffer, pH 7.5 (200 μmol), 0.04 ml 0.1 M NAD (4 μmol), 0.1 ml 0.2 M Na arsenate (20 μmol), 0.05 ml 0.2 M 2-mercaptoethanol (10 μmol), 0.1 ml 0.02 M EDTA, disodium salt (2 μmol), 0.01 ml GAPD (Sigma, from rabbit muscle, about 8 U), and a maximum of 0.1 ml of the neutralized GAP solution, in a total volume of 2.40 ml. The assay was done at 340 nm and room temperature in quartz cuvettes

(4.0 ml capacity). The resulting absorbance change of 3 different dilutions times the dilution factor, divided by 6.22×10^3 equalled the concentration of the stock GAP solution, specifically the concentration of the D-GAP isomer. The concentration of one preparation was 7.0 mM, and three others averaged 12.6 mM (range 12.2-13.4 mM).

Because of the variation in concentration of the GAP solution, and the accompanying variation in volume that contained a given amount of GAP, a standard amount of GAP, 1 μmole, was used in the GAPD assay, as shown in Table 2. This amount may not have been optimal for the enzyme under the conditions of assay and may have been partly responsible for the relatively poor proportionality observed. Nevertheless, reaction rates were linear over the 5-6 min intervals used for calculation of GAPD activity.

7. Calculation of enzyme activity

During each enzyme assay, the recorder chart was set at 1/2" per min and was annotated with the full scale absorbance span and the ratio switch setting. To calculate enzyme activity, the initial linear portion of each curve was scored with initial and final percentages of full scale. If the recording of enzyme activity (represented by a dashed line) was not linear, the best tangent to the line, starting at or near zero time, was used. The difference between the initial and final percentages of full scale multiplied by the full scale absorbance reading equaled the absorbance change caused by the enzyme over a given time interval. The absorbance change divided by the molar absorptivity, a (6.22×10^3) and by the light path (1 cm) equaled the concentration of the reacted coenzyme (equivalent to the substrate) in mol/liter. This multiplied

by the volume of liquid in the cuvette (usually 1.0 ml) x 10^6 μmol/mol yielded the μmol of coenzyme (or substrate) reacted in the time interval. The number of μmol divided by min of reaction time equaled the units of enzyme present, and the units divided by the mg of protein added equaled the specific activity of the enzyme. Because the specific activities were generally low, they were multiplied by 1000 to yield specific activity in milliunits/mg (mU/mg).

In summary:

$$mU/mg = \frac{\text{Fraction of full scale x full scale x vol in cuvette x } 10^6 \text{x} 1000}{6.22 \times 10^3 \text{ l/mole-cm x cm x min x mg}}$$

When there was a significant amount of reduction of coenzyme (generally less than 10% of full scale in 3–4 min) prior to initiation of the reaction, a correction factor was applied to the calculations. This was determined by following the same steps indicated above through division by min, i.e., determining the units of non-specific reduction. These units were then subtracted from the units of enzyme prior to division by mg of protein.

E. Separation and detection of enzymes in polyacrylamide gels

1. Gel electrophoresis

To effect the physical separation of enzymes from each other, crude extracts and ammonium sulfate fractions were subjected to electrophoresis in polyacrylamide gels. The method used was essentially that described by Davis (51). The stock solutions are listed in Table 3.

Twelve glass electrophoresis tubes (I.D. 4 mm, O.D. 6 mm, length 75 mm) were placed into a gel preparation rack (Canalco, Rockville, MD), with a double layer of Parafilm used to close the lower ends of the tubes.

Table 3. Reagents and stock solutions for polyacrylamide gel electrophoresis

SOLUTION A		SOLUTION C1	
1 N HCl	48 ml	Acrylamide	30.0g
Tris base	36.6g	BIS	0.8g
TEMED	0.23 ml	H_2O to	100 ml
H_2O to	100 ml	(for 7.5% gel)	
(pH 8.9)			

SOLUTION C2		SOLUTION E	
Acrylamide	30.0g	Riboflavin	4 mg
BIS	1.6g	H_2O to	100 ml
H_2O to	100 ml		

SOLUTION F		SOLUTION G	
Sucrose	40g	Ammonium persulfate	0.14g
H_2O to	100 ml	H_2O to	100 ml

Abbreviations: TEMED, N,N,N',N'-tetramethylene diamine; BIS, N,N'-methylene-bis-acrylamide. Solutions were prepared in deionized and distilled water and filtered through Whatman No. 1 filter paper into 125 ml brown Nalgene bottles, which were stored at 4C.

Running gel #1 was prepared by mixing 2 ml of solution A (Table 3), 4 ml solution C_1, 8 ml solution G, and 2 ml H_2O in a 50 ml Erlenmeyer flask. Running get #2 consisted of 2 ml solution A, 4 ml solution C_2, 2 ml solution E, and 8 ml H_2O. A 9 in disposable (Pasteur) pipet was used to fill each gel tube, allowing the fluid to flow down the inside of each tube as the pipet was raised along the inside surface, thus avoiding the formation of air bubbles. After adding an appropriate amount of running gel solution, a layer of water 4-5 mm thick was placed atop the gel solution by carefully expelling water from a 2.5 ml syringe (B-D Glaspak; Becton, Dickinson, and Co., Rutherford, NJ) while the bevel was held against the inside surface of the tube. After 30 min of polymerization, the resulting gel column was about 55 mm long. Running gel #2 required photopolymerization, for which the gel tubes were placed 3.5 cm from a Canalco preparation light for 30 min.

Following polymerization, water was drained from the tubes by inverting the rack. The sample was then added in a total of usually 50 µl per tube from either 50 µl disposable pipets (Dade, Miami, FL) or from a 250 µl syringe (Hamilton Co., Reno, Nevada). The sample consisted of cell extract and 40% sucrose (solution F), usually in a 3:1 ratio. The sucrose served a function similar to, and thus replaced, the stacking gel, as described by Dietz (55).

The stacking gel solution consisted of 1 ml solution B, 2 ml solution D, 1 ml solution E, and 4 ml solution F. Solution B consisted of 48 ml 1 N HCl, 5.98 g Tris base, 0.46 ml TEMED, and water to 100 ml. Solution D consisted of 10.0 g acrylamide, 2.5 g BIS, and water to 100 ml. (See Table 3 for explanation of abbreviations.) The stacking gel (0.2 ml) was added after the running gel polymerized. Following photo-

polymerization of the stacking gel, the sample was added as described above. In an experiment which compared gels run with or without a stacking gel, no significant differences in the resulting bands (stained for GAPD activity) were observed. Therefore, the stacking gel was routinely eliminated.

After addition of the sample (extract and sucrose), fresh running gel was prepared, using half the volumes indicated previously. This was carefully placed atop the sample layer in the gel tubes, using a 5 3/4" disposable pipet, followed by a water overlay. After polymerization, the upper gel formed a column 8-10 mm high.

After draining the water, each tube was marked along the running gel at a distance of 40 mm from the bottom of the sample layer. This provided for uniform migration of the marker dye.

Reservoir buffer was prepared by a 1:10 dilution of the stock solution consisting of 6.0 g Tris base, 28.8 g glycine, and H_2O to 1 l, equivalent to 0.05 M Tris - 0.38 M glycine (pH 8.4). About 700 ml of 1 l of diluted buffer was added to the lower reservoir of the electrophoresis cell (constructed by Dr. F. Montiel). The upper end of each gel tube was dipped into this solution to facilitate insertion into the grommets of the upper reservoir. After the 12 tubes were in place, the lower end of each gel was wetted with two drops of lower reservoir buffer to assure proper contact of gel and buffer. The upper reservoir was then inverted and placed on the lower reservoir. The bottoms of the gel tubes were about 5 mm below the surface of the lower reservoir. The remaining 300 ml of diluted buffer was added to the upper reservoir. After eliminating any air bubbles in the tops of the gel tubes, 3 ml of marker dye (0.001% bromphenol blue in water) was added to the upper

reservoir, the top of the electrophoresis cell was added, and the entire unit was placed in a refrigerator (4 C). Connections were made to the power supply (Canalco Electrophoresis Constant Rate Source, model 300 B), the upper reservoir to the negative terminal and the lower reservoir to the positive. After adjusting the current to 2.5 mamp/gel, electrophoresis was allowed to proceed until the marker dye migrated the measured 40 mm, usually about an h. Uneven rates of migration of the marker dye were compensated by removing the gels in which the marker dye had migrated 40 mm, replacing those gel tubes with a short length of glass rod, and readjusting the current to 2.5 mamp/gel. After all marker dyes had migrated the proper distance, gel tubes were removed from the electrophoresis cell and stored in ice prior to staining for enzyme activity. Storage in ice occurred for usually less than 1 h.

Gels were removed from the tubes by ejecting cold water from a 10 ml plastic syringe equipped with a 1 1/4" blunt needle (about 21 gauge) inserted between the gel and the glass, while moving the needle with a circular motion around the inside of the tube.

2. Detection of enzyme activity

Following electrophoresis, polyacrylamide gels were stained for activity of FDP aldolase and GAPD. The procedure for staining FDP aldolase was modified from those of London (175) and Sadoff (251), and several papers on vertebrate aldolases (1,118,187,224). The procedure for GAPD was then derived from the procedure for aldolase.

Enzyme activity was detected directly in the gels by coupling the enzymatic reaction to the reduction of 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'- (3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride

(nitroblue tetrazolium or NBT).

A typical system for assay of FDP aldolase was prepared as follows: 7.5 mg NAD, 0.3 mg phenazine methosulfate (PMS), and 3.0 mg NBT were dissolved in 17.5 ml 0.1 M glycylglycine or HEPES buffer, pH 7.5. Stock solutions of 0.3 or 0.05 M FDP and 0.1 M Na arsenate were also prepared. GAPD and TPI were commercial preparations (from rabbit muscle; Sigma Chemical Co.).

In general, 0.1 ml FDP solution (5 or 30 μmol), 0.2 ml Na arsenate (20 μmol), and water were added to 10 x 75 mm test tubes. Other reagents to be tested were also added, including 15-40 units of GAPD, and 100-200 U of TPI. The two dyes (PMS and NBT) were then added to the buffer (already containing NAD), dissolved, and dispensed, 1.25 ml to each test tube. Each incubation mixture thus contained 1.5 μmol NAD, 0.1 μmol PMS, and 0.5 μmol NBT in a total volume of 1.5-1.6 ml. The final buffer concentration was about 0.08 M. The gels, which had been stored in ice, were removed from the electrophoresis tubes and inserted into the test tubes, where they were almost completely immersed in the incubation fluid. The test tubes were then closed with rubber stoppers and inverted 10-12 times to assure thorough mixing of reagents. The gels were incubated in the dark at 37 C, in either vertical or horizontal position, until adequate color development (dye reduction) occurred, generally 1-1.5 h.

The assay system for GAPD activity was generally similar to that for aldolase. NAD, PMS, and NBT were dissolved in 0.1 or 0.125 M Tris buffer, pH 7.5 or 8.8. The GAP solution, prepared as described previously, was used as the substrate. Sufficient volume of GAP solution was used to provide 1 - 2 μmoles D-GAP per gel. One-tenth ml of 0.1 M

Na arsenate (10 μmol) was added, followed by the freshly prepared buffer-NAD-dye solution (final amounts were 1.5-2.0 μmol NAD, 0.2 μmol PMS, and 0.5 μmol NBT per gel). Other reagents, including 10 or 20 μmol EDTA and 0.1 μmol cysteine were added, in a final volume of 1.6-1.7 ml. Gels were incubated at 37 C in the dark for 1.5-3 h.

After incubation of gels, they were stored in 7% glacial acetic acid solution. The gels were removed from the test tubes and washed briefly in cold tap water. After removing a portion of gel below the marker dye, the gels were inserted into 10 x 63 mm B-D Vacutainer tubes. After filling the tube with 7% glacial acetic acid (from a squeeze bottle) and forming a convex meniscus of acetic acid solution, the rubber stopper was inserted. This was facilitated by inserting a 1" 20 gauge needle through the rubber stopper, then inserting the stopper into the Vacutainer tube, and withdrawing the needle. Such storage in acetic acid preserved the bands of reduced dye and prevented the gels from drying and shrinking.

Bands of enzyme activity were observed with the aid of fluorescent backlighting provided by an Rh View Box (model LL-16115, Thermolyne, Dubuque, Iowa). By measuring the distance of migration of bands and marker dye, the ratio of migration of the bands to the marker dye may be determined, similar to the R_f values in paper chromatography.

F. Studies on the uptake of 2-deoxyglucose

S. aureus was grown in 2% VFCA supplemented with 2 μg each of niacin and thiamine per ml for 17 h, to a cell density of (in the two experiments) 176 and 182 Klett units. The cells were harvested, washed once in 0.05 M K-Pi buffer, pH 7.0, resuspended in 0.05 M K-Pi, pH 7.0,

and distributed in 5-ml aliquots to 250 ml Erlenmeyer flasks, each containing 45 ml of incubation medium. The incubation medium consisted of 0.05 M K-Pi buffer, 0.5 g of 2-deoxyglucose (1% final concentration), plus 2-deoxy-D-glucose-^{14}C-UL (specific activity 6.3 m Ci/mmol; ICN, Irvine, CA). The stock 2-deoxyglucose (2DG) solution was diluted to 5.5 ml in water, which yielded an activity of 1.52×10^7 counts/min (cpm) per ml. (The theoretical activity was 2.02×10^7 disintegrations/min per ml.) To each incubation flask was added 0.5 or 0.4 ml of stock, labeled 2DG, yielding initial theoretical activities of 1.52×10^5 and 1.22×10^5 cpm/ml, respectively. The incubation media also contained other additions, as indicated in the Results section.

At intervals up to 2 h of incubation, aliquots of incubation medium were removed and filtered through membrane filters (either 0.22 μm or 0.45 μm pore size; Millipore Corp., Bedford, MA) contained in a Millipore Sampling Manifold (catalog #3025, 30 sample capacity) with a vacuum supplied by the bench outlet. In the first experiment, the sample size was decreased from 5 to 2 ml to allow more efficient filtration. In the second experiment, using 0.45 μm pore size filters and 2 ml aliquots of incubation medium, complete filtration occurred in 1-1.5 min.

After the initial filtration, the cells were washed with either 5 ml (1st experiment) or 4 ml (2nd experiment) of 0.05 M K-Pi buffer, pH 7.0. (This required 3-4 min in the second experiment.)

The vacuum was released and the filters (and cells) were placed into disposable scintillation vials (Kimble, 27 mm, cap size 25 mm), into which was poured 10 ml of scintillation fluid, prepared as described previously, from a volumetric dispenser (Calab, Berkeley, CA). Each vial was swirled to mix the contents, which caused the filter to

almost completely dissolve. The activities of the samples were determined using a liquid scintillation spectrometer (Packard model 3320), with settings indicated previously.

The results are expressed as counts/min (cpm) per mg dry wt of cells. The cell density in the incubation medium was determined by centrifuging 1-, 2-, and 3-ml aliquots of the original cell suspension in tared 30 ml centrifuge tubes (Corex), determining the wet weight, and multiplying by 0.2 to obtain the dry weight. The cell density in the two experiments was 1.5 and 0.77 mg dry wt/ml. The counts obtained in 10 min were divided by 10 and by the cell density to obtain cpm/mg dry wt.

G. Viability studies

To obtain an approximation of the effect of incubation in PBG on viability of S. aureus, viable counts of S. aureus were made using the pour-plate method. Cells were grown in 2% VFCA supplemented with 2 μg each of niacin and thiamine per ml for 17 h, harvested, washed, suspended in 0.05 M K-Pi buffer, pH 7.0, and incubated in 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose, with and without 30 μmol cysteine-HCl per ml, for 3 h. At hourly intervals, 0.1 ml incubation medium was removed and diluted in water up to 10^{10}-fold. Either 0.1 or 1.0 ml of diluted cell suspension was added to a sterile plastic petri plate, to which was added 15-18 ml sterile TSA (BBL), kept at 40 C in a water bath. After mixing the cells in the media and allowing the agar to solidify, the plates were incubated at 37 C for 18 h. Total colony counts were then determined.

For a second method of obtaining a rough estimate of the effects of incubation on viability of S. aureus, one loopful of incubation medium

was streaked onto one-half of a TSA plate and incubated overnight at 37 C. Although this method certainly was not quantitative, the almost constant density of cells in a given set of incubation flasks and the one loopful of culture streaked allowed a comparison to be made between incubation of S. aureus in PBG only and in PBG plus cysteine and other sulfhydryl reagents.

H. Sources of chemicals

Toluene and chloroform were scintillation grade from Eastman Kodak Co., Rochester, NY or Fisher Scientific Co., Fairlawn, NJ. Other scintillation chemicals, except PPO, were from Fisher. PPO was obtained from Eastman. Radioactive glucose was obtained from Amersham-Searle Co., Arlington Heights, IL, or from ICN, Irvine, CA. 2-Deoxyglucose - ^{14}C was from ICN.

Acrylamide, BIS, ammonium persulfate, and TEMED, for gel electrophoresis, were obtained from Bio-Rad Laboratories, Richmond, CA. Riboflavin was obtained from Merck and Co., Rahway, NJ.

All enzymes, substrates, and buffers, except phosphate, as well as amino acids, anthrone, and sucrose, were obtained from Sigma Chemical Co., St. Louis, MO.

All other reagents were analytical reagent grade obtained from Mallinckrodt Chemical Works, St. Louis, MO. Monosodium and disodium phosphate were obtained from J.T. Baker Chemical Co., Phillipsburg, NJ.

RESULTS

I Effects of incubation of S. aureus in phosphate-buffered glucose

A. Levels of metabolic intermediates

1. Levels of intermediates in growing cells

The intracellular levels of several metabolic intermediates were measured in phenol extracts prepared after the 17-h growth period of S. aureus. These are termed zero-time levels and are so indicated in the tables. The levels of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), pyruvate, and 6-phosphogluconate (6PG) were consistently at a concentration of less than 1 μmol/g dry wt in growing cells. The only Embden-Meyerhof (EM) pathway intermediate that was consistently higher was phosphoenolpyruvate (PEP), at a concentration of 1-2 μmol/g at zero time.

The levels of nicotinamide adenine dinucleotide (NAD) were 3-7 μmol/g, depending on the growth medium. In one experiment the level of NAD in cells grown with 2 μg each of niacin and thiamine was 4.6 μmol/g, but was only 0.2 μmol/g when vitamins were eliminated from the Vitamin-Free Casitone (VFC) growth medium. A similar experiment yielded 3.7 μmol NAD/g with both vitamins and 0.1 μmol NAD/g when only thiamine was present during growth. The level of nicotinamide adenine dinucleotide phosphate (NADP) was consistently less than 1 μmol/g at zero time, ranging from 0.4-0.9 μmol/g in cells grown in VFC and 0.2-0.5 μmol/g in cells grown in Vitamin-Free Casamino Acids (VFCA). The ratio of NAD/NADP in cells grown in VFC was about 6:1 (average of 6; range

4.4-7.5), and in cells grown in VFCA, about 19:1 (average of 3; range 12-28). These results refer only to NAD and NADP and do not include NADH or NADPH.

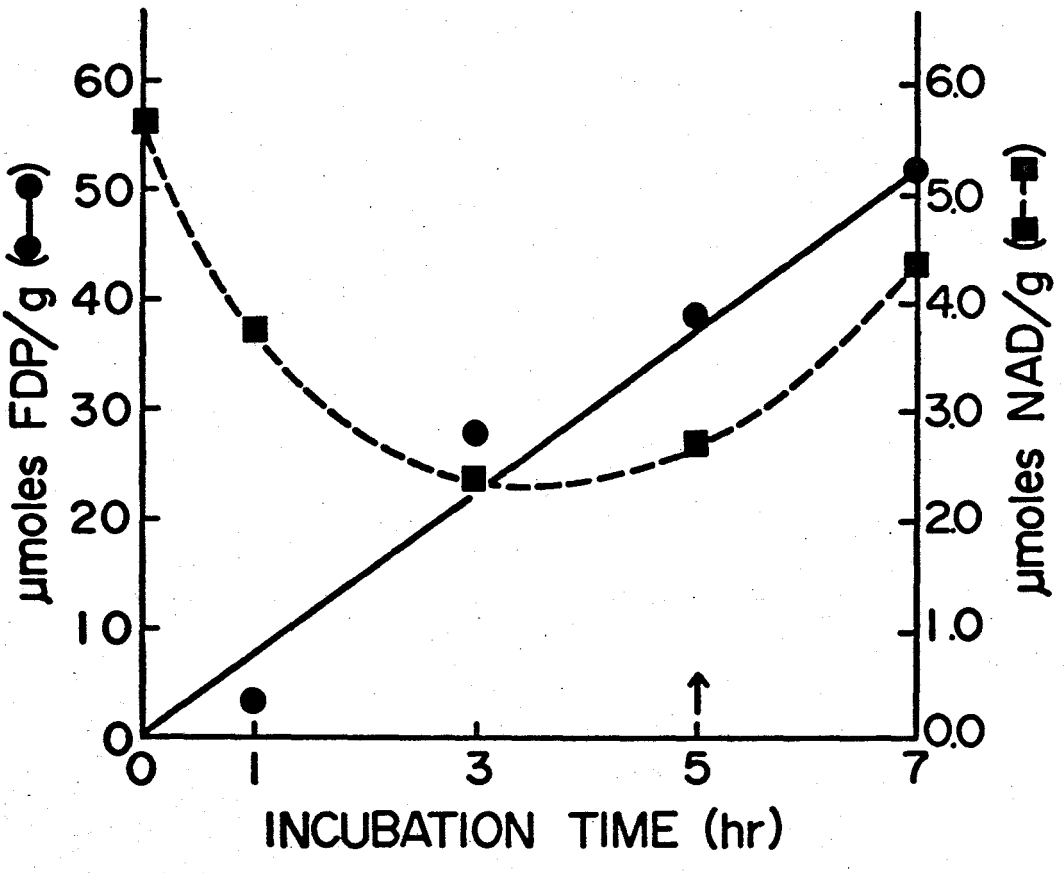
The metabolite with the highest zero time level was adenosine-5'-triphosphate (ATP). In two early experiments, with cells grown in VFC with 2 μg each of niacin and thiamine (turbidity 310 Klett units), the level of ATP was 8.3 and 13.0 (average of 10.6) μmol/g, and in cells grown in VFC with 10 μg niacin and 4 μg thiamine (345 Klett units), ATP was at 12.7 and 14.3 (average of 13.5) μmol/g.

The presence of glucose during growth did not affect the level of FDP. In an experiment in which 0.25% glucose was added directly to the medium or via a diffusion capsule (228) containing 30% glucose, the levels of FDP after 12 h were both < 1.0 μmol/g. The NAD concentrations were 5.8 and 6.4 μmol/g, respectively. After 12 h, the cells grew to densities of 319 and 324 Klett units, respectively. The corresponding activities of the HMP pathway were 25.4% and 24.1%.

2. Changes in levels of intermediates during incubation of *S. aureus* in phosphate-buffered glucose

One purpose of this investigation was to determine a possible relationship between the activity of the HMP pathway and the levels of intracellular intermediates, particularly FDP. *S. aureus* was incubated in phosphate-buffered glucose (PBG; 0.05 M potassium phosphate buffer, pH 7.0, plus 1% glucose unless otherwise indicated) for periods of up to 6 h, and levels of intermediates were measured in phenol extracts of the cells. In general, the levels of all intermediates, except PEP and NAD, increased during incubation. Figure 1 shows the results of

Figure 1. Levels of FDP and NAD in S. aureus during incubation in phosphate-buffered glucose



S. aureus was grown in 2% VFC plus 10 µg niacin and 4 µg thiamine per ml (325 Klett units), washed twice in 0.8% NaCl, and incubated in 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose, 100 µg 6-aminonicotinamide/ml and 2 µg thiamine/ml. After 5 h of incubation (arrow), niacin was added to a final concentration of 100 µg/ml. Cell density during incubation was 0.12 g dry wt/100 ml; 500 ml total volume. At intervals, phenol extracts were prepared for assay of FDP and NAD. The data presented here were obtained from one experiment.

one incubation in which the level of FDP increased from < 1 to more than 50 μmol/g after 7 h. In this same time, NAD decreased from 5.6 to 2.4 μmol/g, and then increased to 4.4 μmol/g after niacin was added to the incubation medium. The addition of niacin apparently did not affect the increase in the level of FDP.

Although the values reported in the tables are based on individual incubation flasks from single experiments, the results of duplicate assays of the individual extracts were very reproducible, ± 2% or better. An experiment in which duplicate flasks were incubated and extracted in parallel also yielded similar FDP values, for example, 50.6 ± 1.2 μmol/g.

B. Effect on the glucose catabolic pathways

Estimation of pathway participation in non-growing S. aureus indicated that cells that had grown for 17 h catabolized 18-25% of the glucose via the HMP pathway. The remainder of the glucose was catabolized by the EM pathway. After incubation of cells in PBG, the % HMP generally decreased slightly, and, as will be presented in subsequent tables, additions to the incubation medium caused greater changes in the HMP pathway. The percentage of glucose utilized by the cells during the estimation, as well as the amount catabolized by the HMP pathway, also consistently decreased after incubation in PBG with or without additions. After 17 h of growth in VFC, the percentage of glucose used by the non-growing cells was 79.5 ± 7.3% at zero time (in 6 experiments) and 52.9 ± 1.9% after 6 h of incubation in PBG (4 experiments).

Table 4 presents a summary of the levels of intermediates in S. aureus during incubation in PBG. The cells were incubated and washed under standard conditions, and the data represent those obtained from

Table 4. Intracellular levels of some glycolytic intermediates in S. aureus after growth in VFC or VFCA and subsequent incubation in phosphate-buffered glucose

Intermediate	Incub. time, h	Conc., μmol/g Mean level	± S.D.	Range	No. of Expts.
Cells grown in VFC[a]					
G6P	3	1.6	0.35	1.2-2.2	6
	6	2.2	0.50	1.7-2.9	5
F6P	3	2.7	0.64	1.8-3.3	4
	6	2.8	0.76	1.9-3.3	3
FDP	3	87.3	17.3	59.1-113.0	14
DHAP	3	3.7	1.08	1.6-5.6	14
PEP	0	1.7	0.46	1.1-2.3	8
6PG	3	1.2	0.21	1.0-1.4	4
	6	2.0	1.74	1.0-5.5	6
NAD	0	3.6	0.62	2.9-5.2	14
	3	2.5	0.58	1.5-3.4	13
	6	1.8	0.63	1.2-2.8	7
ATP	0	16.3	5.0	10.6-19.3	3
	3	27.1	1.5	25.8-28.7	3
Cells grown in VFCA[a]					
FDP	3	116.1	38.6	60.4-177.7	10
DHAP	3	3.0	1.54	1.4-5.3	9
NAD	0	7.0	1.28	4.0-8.8	11
	3	4.5	0.88	2.4-5.8	11
ATP	0	7.6	3.3	4.1-10.6	3
	3	20.9	6.1	15.0-27.2	3

[a]S. aureus was grown in either 2% VFC or 2% VFCA plus 2 μg each of niacin and thiamine for 17 h, washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in 0.05 M K-Pi buffer, pH 7.0, plus 1.0% glucose. After 3 or 6 h of incubation, cells were washed once before phenol extracts were prepared for spectrophotometric assay of intermediates.

control flasks in the indicated number of experiments. The glucose used after growth in VFCA were 65.3 ± 4.1% at 0 time and 54.3 ± 6.0% after 3 h of incubation in PBG (9 experiments). This decrease was caused by a slight reduction in the actual amounts of glucose utilized by both pathways. As will be shown in the next several tables, it was apparently the HMP pathway that was more severely affected by incubation, both on a percentage basis and in the amount of glucose used by the pathway. Although the percent of glucose used by the EM pathway increased as the percent HMP decreased, the actual amount of glucose used by the EM pathway was relatively unaffected by the various incubation conditions (Tables 6-9).

II Lack of effect of FDP on the HMP pathway; effects of 6-aminonicotinamide

A major purpose of this investigation was to examine the possible effects of FDP on glucose catabolism via the HMP pathway. It had been found by Wadke (306) that incubation of S. aureus in 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose caused a reduction in the percentage of glucose catabolized by the HMP pathway. This section will describe the results of experiments using this finding, and the observation that the EM pathway intermediate FDP accumulated to very high intracellular levels under the same conditions. The niacin analog, 6-aminonicotinamide (6AN), was also used to effect specific changes in the HMP pathway.

A. Effects of 6-aminonicotinamide on levels of 6-phosphogluconate

S. aureus was incubated in 0.05 M K-Pi buffer, pH 7.0, with or without glucose (1%) and with or without 6AN at 100 μg/ml. The results in Table 5 show increases in the levels of all intermediates tested, except NAD. 6PG increased slightly more in the presence of 6AN. Apparent from experiment B are that no accumulation of EM intermediates or of 6PG occurred in the absence of glucose and that glucose tended to accelerate the decrease in the level of NAD. In part B, the respective decreases in NAD at 6 h were 58%, 52%, 6%, and 13%.

To enhance any possible effects of 6AN on the intermediates, S. aureus was incubated in PBG with concentrations of 6AN ranging from 0 to 2500 μg/ml (Table 6). FDP increased at 3 h and then seemed to decrease after 6 h. There were no apparent effects of 6AN on the EM intermediates. In contrast, the increase of 6PG was directly related to the concentration of 6AN in the incubation medium.

After observing the apparent discrepancy in the levels of FDP in

Table 5. Effects of low concentration of 6-aminonicotinamide on levels of intermediates during incubation of S. aureus

Expt.	Incubation Medium	Incub. Time, h	Intermediates, μmol/g						
			G6P	F6P	FDP	DHAP	6PG	NAD	ATP
A	PBG+Thiamine +6AN	0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	4.1	19.3
		3	< 1.0	1.9	117.8	1.6	1.6	2.6	26.6
		6	1.0	2.2	147.2	< 1.0	1.9	2.0	28.1
	PBG+Thiamine	3	1.0	1.7	110.0	1.2	1.2	3.4	27.8
		6	< 1.0	1.5	131.1	1.8	< 1.0	2.5	30.5
	PBG	3	< 1.0	1.8	101.3	1.8	1.4	3.4	26.7
		6	< 1.0	1.9	141.7	2.0	1.0	2.8	31.1
B	PBG+6AN	0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	5.2	nd
		3	< 1.0	3.6	118.1	8.7	1.0	3.1	nd
		6	1.3	3.7	129.3	5.4	2.8	2.2	nd
	PBG	3	< 1.0	3.3	106.4	6.3	< 1.0	3.4	nd
		6	< 1.0	3.3	104.3	4.8	1.5	2.5	nd
	Buffer+6AN	3	nd	nd	< 1.0	< 1.0	< 1.0	5.1	nd
		6	< 1.0	< 1.0	< 1.0	1.6	< 1.0	4.9	nd
	Buffer only	3	< 1.0	< 1.0	< 1.0	2.9	< 1.0	4.6	nd
		6	< 1.0	< 1.0	< 1.0	1.6	< 1.0	4.5	nd

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h, washed twice in 0.8% NaCl, re-suspended in 0.05 M K-Pi buffer, pH 7.0, and added to the indicated incubation media. Phenol extracts were prepared at 0,3, and 6 h for spectrophotometric assay of intermediates. PBG, 0.05 M K-Pi buffer, pH 7.0 + 1% glucose. Thiamine, final concentration 2 μg/ml. 6AN, 6-aminonicotinamide, final concentration 100 μg/ml. nd, not done. Cell densities during incubation were: Part A, 0.14-0.15 g dry wt/100 ml; Part B, 0.15-0.19 g dry wt/100 ml, in a total of 300 ml. The data are from two expts.

Table 6. Effects of high concentrations of 6-aminonicotinamide during incubation on pathways and intermediates in S. aureus

Addition to PBG Incubation Medium	Incub. Time, h	% HMP	% EM	Glucose used, μmol per h	Glucose used, μmol via HMP	Glucose used, μmol via EM	Incub. Time, h	Intermediates, mol/g G6P	F6P	FDP	DHAP	6PG	NAD
None	0	19.6	80.4	8.1	1.6	6.5	0	<1.0	<1.0	<1.0	<1.0	<1.0	3.4
							3	1.2	2.9	97.1	1.6	1.0	2.4
	6	20.8	79.2	6.2	1.3	4.9	6	1.8	3.1	62.5	1.6	1.1	1.7
100 μg 6AN/ml							3	<1.0	3.0	90.7	3.1	2.4	2.1
	6	15.6	84.4	5.8	0.9	4.9	6	2.0	3.0	52.7	1.2	4.1	1.4
500 μg 6AN/ml							3	<1.0	3.0	102.2	2.4	7.2	1.8
	6	12.4	87.6	6.6	0.8	5.8	6	1.8	2.6	63.9	1.0	21.3	1.1
2500 μg 6AN/ml							3	<1.0	2.3	79.2	2.4	28.5	2.0
	6	8.5	91.5	5.7	0.5	5.2	6	1.7	2.5	43.2	1.2	81.5	1.1

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h, washed twice with 0.05 M K-Pi buffer, pH 7.0, resuspended in the same buffer, and added to the indicated incubation media. At 0, 3, and 6 h, pathways were estimated and phenol extracts were prepared as described in Methods. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. 6AN, 6-aminonicotinamide. Cell density was 0.14-0.19 g dry wt/100 ml, in a total incubation volume of 300 ml. The data are from one experiment. Note: for the 3 h extraction, cells were washed once in buffer. At 6 h, cells were washed three times.

these cells, i.e., FDP increased for 3 h, then decreased after 6 h, it was realized that the cells had been washed once for the 3 h phenol extraction and three times for the 6 h extraction. This suggested that washing the cells in buffer might remove some of the accumulated intermediates. To test this hypothesis, S. aureus was incubated in PBG plus 0, 500, and 2500 μg 6AN/ml for 6 h, then washed 3 times. A phenol extract was prepared after each wash, and the results of the assays are shown in Table 7. The greatest effect of washing occurred with FDP, which had accumulated to very high intracellular levels, and with 6PG, which again accumulated in proportion to the concentration of 6AN. The results of this experiment suggested that high intracellular levels of FDP and 6PG were subject to elution from the cells by washing. From these results, the effects of washing the cells became more apparent, and in subsequent experiments, cells were washed once in a constant volume of buffer.

To further demonstrate the effects of 6AN on intermediates, S. aureus was incubated in 0.05 M K-Pi buffer, pH 7.0, with or without 1% glucose and with or without a low or high concentration of 6AN. The results, in Tables 8 and 9, indicated that FDP increased to very high intracellular levels in PBG and that 6AN apparently caused an increase in 6PG. The results in both Tables 8 and 9, each representing one experiment, showed again that intermediates did not accumulate in the absence of glucose, and that both glucose and 6AN accelerated the decrease of NAD.

Measurement of glucose utilization in the experiment represented by Table 9 indicated that the cells incubating in PBG plus 2500 μg

Table 7. S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (average Klett units 319), washed twice with 0.05 M K-Pi buffer, pH 7.0 and incubated in the indicated media for 6 h. Total incubation volume 300 ml. Pathways were estimated at 0 and 6 h. For assay of intermediates, cells were treated in the following way: The incubation mixtures were centrifuged, an aliquot of supernatant fluid was removed before decanting, and the cells were resuspended in buffer. From this suspension, an aliquot was removed to be centrifuged and extracted with phenol. This process was repeated three times, thus yielding cells that had been washed 0, 1, 2, and 3 times, respectively.

For estimation of pathways at zero time, cells were washed twice in 0.05 M K-Pi buffer, pH 7.0. For the 6 h estimation, a portion of cells was removed during the first repeat of the washing procedure, thus yielding cells that were washed once.

Cell density during incubation was about 0.11-0.13 g dry wt/100 ml in a total volume of 300 ml. (One experiment.)

Table 7. Effects of high concentrations of 6-aminonicotinamide on pathways and intermediates in S. aureus; the effect of washing of cells on levels of intermediates

Addition to PBG Incubation Medium	Incub. Time h	% HMP	% EM	Glucose used, μmol/g cells per h	via HMP	via EM
None	0	22.8	77.2	9.1	2.1	7.0
500 μg 6AN/ml	6	13.7	86.3	5.8	0.8	5.0
2500 μg 6AN/ml	6	7.4	92.6	6.5	0.5	6.0

Sample	Incubation Medium (0.05 M K-Pi buffer, pH 7.0, plus 1% glucose) and No Further Additions				500 μg 6AN/ml				2500 μg 6AN/ml			
	μmol -/g cells				μmol -/g cells				μmol -/g cells			
	G6P	FDP	6PG	NAD	G6P	FDP	6PG	NAD	G6P	FDP	6PG	NAD
Pre-Incubation (zero time)	<1.0	<1.0	<1.0	3.1	<1.0	<1.0	<1.0	3.1	<1.0	<1.0	<1.0	3.1
Post-Incub.	2.5	208.0	1.4	1.4	1.8	192.0	33.3	<1.0	2.9	168.7	93.6	<1.0
Wash No. 1	1.9	172.6	<1.0	1.3	1.5	177.2	26.1	<1.0	1.3	151.0	84.3	<1.0
Wash No. 2	1.8	115.0	<1.0	1.3	1.5	126.5	6.3	<1.0	1.9	95.5	31.3	<1.0
Wash No. 3	2.2	107.6	<1.0	1.6	2.1	113.5	4.8	<1.0	2.0	122.6	39.7	1.0
% Change During Washing	-12	-49	-28	+14	+17	-41	-86	0	-31	-27	-58	+11

Table 8. Effects of glucose and/or low concentration of 6-aminonicotinamide on pathways and intermediates in S. aureus.

Incubation Medium	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM
None	0	18.0	82.0	6.5	9.8	1.8	8.0
PBG + 6AN	6	16.8	83.2	2.1	8.0	1.3	6.7
PBG	6	17.6	82.4	2.6	7.2	1.3	5.9
Buffer + 6AN	6	18.3	81.7	3.3	7.6	1.4	6.2
Buffer only	6	25.5	74.5	3.2	5.8	1.5	4.3

Incubation Medium	Incub. Time, h	Intermediates, μmol/g G6P	FDP	DHAP	6PG	NAD
None	0	<1.0	<1.0	<1.0	<1.0	3.3
PBG + 6AN	3	1.2	113.3	4.3	9.0	1.7
	6	1.4	118.4	3.2	42.4	1.4
PBG	3	1.6	106.5	2.5	1.1	1.7
	6	1.7	152.9	2.3	5.5	1.5
Buffer + 6AN	3	<1.0	<1.0	<1.0	<1.0	3.2
	6	<1.0	<1.0	<1.0	<1.0	2.3
Buffer only	3	<1.0	<1.0	<1.0	<1.0	3.7
	6	<1.0	<1.0	<1.0	<1.0	2.8

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine for 17 h (325 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. Pathways were estimated at 0 and 6 h. Cells were washed once before phenol extracts were prepared at 0, 3, and 6 h for spectrophotometric assay of intermediates. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. 6AN, 6-aminonicotinamide, 100 μg/ml. Cell density was 0.09-0.12 g dry wt per 100 ml; 300 ml total volume. (One experiment.)

Table 9. Effects of glucose and/or high concentration of 6-aminonicotinamide on pathways and intermediates in S. aureus

Incubation Medium	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM
	0	22.4	77.6	10.7	7.9	1.8	6.1
PBG+6AN	3	9.1	90.9	4.7	7.6	0.7	6.9
	6	4.3	95.7	1.3	7.0	0.3	6.7
PBG	3	25.4	74.6	5.9	5.3	1.3	4.0
	6	13.1	86.9	4.3	5.0	0.7	4.3
Buffer+6AN	3	24.8	75.2	6.3	5.7	1.4	4.3
	6	12.5	87.5	4.6	5.6	0.7	4.9
Buffer only	3	18.4	81.6	6.8	8.8	1.6	7.2
	6	14.8	85.2	4.4	6.9	1.0	5.9

Incubation Medium	Incub. Time, h	Intermediates, μmol/g G6P	FDP	DHAP	PEP	6PG	NAD
	0	<1.0	<1.0	<1.0	1.8	<1.0	3.4
PBG+6AN	3	<1.0	79.4	2.2	<1.0	18.2	1.4
	6	2.7	105.3	1.8	<1.0	104.6	1.0(71)
PBG	3	1.5	107.6	1.8	<1.0	<1.0	2.1
	6	2.9	104.3	1.6	<1.0	<1.0	1.3(62)
Buffer+6AN	3	<1.0	<1.0	<1.0	1.8	<1.0	2.9
	6	<1.0	<1.0	<1.0	1.0	<1.0	2.1(38)
Buffer only	3	<1.0	<1.0	<1.0	1.6	<1.0	3.3
	6	<1.0	<1.0	<1.0	<1.0	<1.0	2.8(18)

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine for 17 h (233 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. Pathways were estimated and phenol extracts were prepared at 0, 3, and 6 h. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. 6AN, 6-aminonicotinamide, 2500 μg/ml. Cell density was 0.11-0.18 g dry wt/100 ml; 300 ml total incubation volume. (One experiment.)

6AN/ml used about 6.3% of the glucose, but the cells in PBG alone used about 15.3%. This suggested that 6AN inhibited glucose utilization by the non-growing cells.

To summarize, the level of FDP in non-growing S. aureus increased to over 200 μmol/g after 6 h of incubation in PBG. Other EM pathway intermediates underwent much smaller increases, except PEP, which decreased during incubation. Only the HMP pathway intermediate 6PG accumulated in direct relation to the concentration of 6AN in the incubation medium. It was also observed in these experiments that glucose considerably accelerated the decrease in the level of NAD during incubation.

B. Effects of 6-aminonicotinamide on the HMP pathway; comparison with levels of FDP

As shown in Tables 6-9, the glucose catabolic pathways in the non-growing cells were estimated at times corresponding to the phenol extractions, plus 2 h of incubation during the estimation, thus allowing comparisons between intracellular levels of intermediates and participation of the pathways. The data in Table 6 showed that incubation of S. aureus in PBG plus 0-2500 μg 6AN/ml resulted in high and similar accumulations of FDP, and accumulations of 6PG that were directly related to the concentration of 6AN. The % of glucose catabolized by the HMP pathway was inversely related to the concentration of 6AN, decreasing from 20.8% with no 6AN to 8.5% with 2500 μg 6AN/ml, after 6 h of incubation. The μmol of glucose utilized by the HMP pathway also showed a decrease in relation to the % HMP pathway. Although the % EM pathway increased with increasing 6AN after 6 h, measurement of glucose utilization showed that the actual μmol catabolized by the EM pathway

remained essentially constant after 6 h. A second experiment yielded similar results (Table 7). FDP increased to high levels in PBG, 6PG accumulated in direct relation to the concentration of 6AN, and the HMP pathway decreased in inverse relation to 6AN. Again, the decrease in the % HMP pathway was reflected in the decrease in the μmol of glucose catabolized by this pathway.

These two experiments indicate that FDP accumulated during incubation in PBG and that 6AN apparently specifically inhibited the HMP pathway, the latter manifested by the accumulation of 6PG and the decrease in the activity of the HMP pathway.

The experimental design presented in Tables 8 and 9 conveniently allowed the following two comparisons: (i) elimination of glucose from the incubation medium prevented the accumulation of FDP and any other EM pathway intermediate; (ii) addition of 6AN caused the accumulation of 6PG and the corresponding decrease in activity of the HMP pathway.

Incubation of S. aureus in 0.05 M K-Pi buffer, pH 7.0, with or without 1% glucose and with or without 2500 μg 6AN/ml resulted in large changes in both the percentage and in the amount of glucose catabolized via the HMP pathway (Table 9). The % HMP decreased by almost one-half in 3 sets and by 81% in PBG + 2500 μg 6AN/ml. The μmol of glucose utilized by the HMP pathway were significantly reduced in all 4 sets, whereas the amount of glucose used by the EM pathway was relatively constant.

During incubation without 6AN, the absence of accumulation of 6PG in the presence of high levels of FDP (Tables 8 and 9) indicated that 6PG dehydrogenase was not significantly inhibited in vivo.

C. FDP levels and the HMP pathway: lack of relationship

The data in Tables 6-9 provide evidence that, in the systems used here, high levels of FDP apparently did not significantly inhibit the activity of the HMP pathway. In Table 6, after 6 h of incubation with increasing concentrations of 6AN, the levels of FDP were high and varied over a 20.7 μmol/g range (from a low of 43.2 μmol/g to a high of 63.9), but under the same conditions, the activity of the HMP pathway decreased from 20.8% to 8.5% at the highest concentration of 6AN. If FDP inhibited the HMP pathway, then cells with the lowest level of FDP (43.2 μmol/g) would not be expected to have the lowest HMP activity (8.5%). Similar results occurred in the experiment represented by Table 7, with high, relatively close values for FDP (192.0 and 168.7 μmol/g) and lower, but significantly different activities of the HMP pathway (13.7 and 7.4%, respectively).

In Table 8 are shown two sets of vastly different levels of FDP, one greater than 118 μmol/g and the other <1.0 μmol/g. However, the HMP pathway in these cells was very similar in 3 sets (16.8-18.3%) and not very different in the fourth (25.5%). Similarly in Table 9, the levels of FDP at 6 h were either very high (over 100 μmol/g) or very low (<1.0 μmol/g), but again, the corresponding activities of the HMP pathway were similar in 3 sets (12.5 - 14.8%) and very low in the fourth (4.3%). Data in Tables 6, 7, and 9 may be used to analyze the effect of 6AN on the HMP pathway. The zero time activity of the HMP pathway in 3 experiments was 21.6 $\pm$ 1.7%. After 6 h of incubation in the presence of 500 or 2500 μg 6AN/ml, the activity of the HMP pathway was 13.0 $\pm$ 0.9% and 6.7 $\pm$ 2.2% in 2 and 3 experiments, respectively. Therefore, both concentrations of 6AN had highly significant ($p < 0.001$) inhibitory

effects on the HMP pathway.

Analysis of the pathways data yielded the following: For cells grown in VFC and incubated in PBG, the activity of the HMP pathway after 0 and 6 h of incubation was 19.7 $\pm$ 2.2% (5 experiments) and 18.8 $\pm$ 4.5% (4 experiments), respectively. With FDP levels of $<$ 1.0 and 107.6 $\pm$ 37.0 μmol/g in the 4 experiments after 6 h of incubation ($p < 0.01$), there was no significant effect of such high levels of FDP on the activity of the HMP pathway ($p < 0.8$).

For cells grown in VFCA and incubated in PBG, the activity of the HMP pathway after 0 and 3 h of incubation was 21.7 $\pm$ 3.4% and 17.4 $\pm$ 3.4%, respectively, in 9 experiments. The levels of FDP at 0 and 3 h of incubation were $<$ 1.0 and 116.8 $\pm$ 34.1 μmol/g (9 experiments). This was a significant increase in the level of FDP ($p < 0.001$) and there was apparently a statistically significant reduction in the activity of the HMP pathway ($p < 0.02$). However, as will be discussed, other factors probably acted to influence the activity of the HMP pathway.

In summary, these results indicate that there was no apparent relationship between the intracellular concentrations of FDP and the activity of the HMP pathway. Furthermore, the lack of accumulation of 6PG in the presence of high levels of FDP indicated that FDP apparently did not inhibit 6PG dehydrogenase in vivo. Other observations are that 6AN had significant and specific effects on the HMP pathway and on the levels of 6PG, but apparently did not significantly nor consistently influence the accumulation of FDP. The HMP pathway was much more variable than the EM pathway, as the actual amount of glucose utilized by the former was lowered considerably by 6AN while that metabolized via the EM pathway

remained relatively constant under all incubation conditions, even in the presence of extremely high intracellular concentrations of FDP.

III Factors affecting the accumulation of FDP in non-growing S. aureus

The second phase of this investigation was directed toward elucidation of some of the factors causing the unusual accumulation of FDP. A number of factors were found, most of which exerted their influence during the actual incubation of the organisms. Several factors, however, were active during the growth of the cells and affected the accumulation of FDP during the subsequent incubation. These will be presented first.

A. Factors acting during the growth of S. aureus

1. Niacin

It had been observed that a decrease in the HMP pathway was accompanied by a parallel decrease in the level of NAD (127). Also, growth with a low concentration of niacin resulted in lower activity of the HMP pathway (17). The purpose of this experiment was to repeat the observations regarding the parallel decrease in HMP pathway activity and NAD concentration, and to test the possible relation of the accumulation of FDP to low HMP pathway activity. Although previous experiments had suggested that FDP did not affect the HMP pathway, it was possible that low HMP pathway activity might influence the accumulation of FDP. Accordingly, S. aureus was grown with low (0.1 μg/ml) and higher (2 μg/ml) concentrations of niacin. The results (Table 10) showed differences in both pathways and intermediates. The % HMP pathway was lower in the cells grown with 0.1 μg niacin/ml. Although the total glucose used was similar at each time (10.0 vs. 10.2, etc.), more was catabolized via the HMP pathway in the cells grown with the higher niacin concentration at 0 and 6 H incubation. FDP accumulated to a much higher level in the cells grown with the lower niacin concentration. This suggests that more

Table 10. Effects of niacin concentration during growth on pathways and intermediates during subsequent incubation of S. aureus

Vitamin Supplements	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, µmol per h	Glucose used, µmol via HMP	Glucose used, µmol via EM	Intermediates, µmol/g G6P	Intermediates, µmol/g FDP	Intermediates, µmol/g DHAP	Intermediates, µmol/g PEP	Intermediates, µmol/g NAD
0.1 µg niacin	0	14.6	85.4	3.9	10.0	1.5	8.5	<1.0	<1.0	<1.0	1.5	<1.0
and 2 µg	3	10.2	89.8	5.0	6.8	0.7	6.1	<1.0	93.6	4.2	<1.0	<1.0
thiamine per ml	6	11.4	88.6	5.6	5.8	0.7	5.1	<1.0	204.4	6.0	<1.0	<1.0
2 µg each of	0	21.3	78.7	10.3	10.2	2.2	8.0	<1.0	<1.0	<1.0	2.3	3.0
niacin and	3	13.0	87.0	11.2	6.2	0.8	5.4	2.2	87.0	3.8	1.2	1.5
thiamine	6	23.7	76.3	6.6	5.9	1.4	4.5	2.3	110.6	3.1	1.0	1.2

S. aureus was grown in 2% VFC plus the indicated vitamin supplements for 17 h. (Final cell densities were 306 Klett units with 0.1 µg niacin/ml and 291 Klett units with 2 µg niacin/ml). Cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose. At the times indicated, pathways were estimated and phenol extracts were prepared. Cell density during incubation was 0.26-0.28 g dry wt/100 ml in 300 ml total volume. (One experiment.)

glucose was catabolized via the EM pathway under these circumstances, which was confirmed by measurement of the actual glucose utilization.

Therefore, these results suggested that the activity of the HMP pathway indirectly affected the accumulation of FDP, possibly by shunting more glucose into the EM pathway and thus supplying more FDP for accumulation. The relatively high and low levels of NAD in these cells corresponded to the media in which they were grown, i.e., with respective high and low concentrations of niacin.

2. Thiamine

Because of the requirement for thiamine for operation of the tricarboxylic acid (TCA) cycle in staphylococci (17), S. aureus was grown in VFC plus niacin, with and without thiamine, to test the possible effect of TCA cycle activity on the accumulation of FDP. The relatively high activity of the TCA cycle in cells grown in TSB (12.4 in one experiment) allowed comparison of a wide range of TCA cycle activities. The results of two separate experiments, in Table 11, indicated that lack of thiamine inhibited the accumulation of FDP, with levels of 66.1 and 76.4 μmol/g in cells grown with thiamine and incubated in buffer plus 1% glucose, but only 5.0 and 3.0 μmol/g in cells grown without thiamine and similarly incubated. The mean values are 71.2 ± 7.3 and 4.0 ± 1.4 μmol/g, respectively ($p < 0.01$).

The total lack of accumulation of FDP in the cells grown in TSB (which contains glucose) was unexpected. This suggested that growth of cells in the presence of glucose and/or phosphate may have been partially responsible. But since TSB is a much richer medium than VFC, the presence of other unknown factors, such as vitamins and/or salts, may have

Table 11

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h in the indicated growth media. The numbers in parentheses below each growth medium are the 17 h cell densities in Klett units, K.U. The cells were harvested, washed once in 0.1 M K-Pi buffer, pH 7.0, and incubated in 0.1 M K-Pi buffer, pH 7.0, plus the indicated additions. Incubation times were: part A, 2 h, except for cells grown in TSB (2 1/2 h); part B, 3 h. Phenol extracts were prepared at zero time (indicated by "None" under incubation medium) and at the end of the incubation period for spectrophotometric assay of intermediates. Amino acids and their final concentrations: arginine, 0.05 M, cysteine, 0.03 M, and glutamine, 0.2 M. The ammonium phosphate added (part B) resulted in final concentrations of 0.05 and 0.5 M NH_4+, but, because of the Pi contributed by the buffer (0.1M), the final concentrations of Pi were 0.15 M and 0.6 M, respectively. In part B, PBG refers to 0.1 M K-Pi buffer, pH 7.0, plus 1% glucose. Cell densities during incubation were: part A, 0.09-0.10 g dry wt for cells grown with niacin and thiamine, 0.04-0.05 g dry wt for cells grown with niacin only and in TSB; part B, 0.10-0.15 g dry wt for cells grown with niacin and thiamine, 0.04 g dry wt for cells grown with niacin only, in 100 ml total volume. (Two experiments.)

Table 11. Effects of growth medium, and effects of glucose and amino acids during incubation, on the accumulation of FDP

Growth Medium	Incubation Medium	Intermediates, μmol/g FDP	DHAP	PEP	Pyruvate
(A) VFC + 2μg niacin and 2μg thiamine (196 K.U.)	None	<1.0	<1.0	1.3	<1.0
	Buffer only	<1.0	<1.0	1.1	<1.0
	+0.1% glucose	31.9	1.8	<1.0	<1.0
	+1.0% glucose	66.1	3.6	<1.0	<1.0
	+5.0% glucose	71.6	3.1	<1.0	<1.0
VFC + 2μg niacin (162 K.U.)	None	<1.0	<1.0	2.6	7.5
	Buffer only	<1.0	<1.0	---	---
	+0.1% glucose	4.8	<1.0	---	---
	+1.0% glucose	5.0	<1.0	---	---
	+5.0% glucose	6.5	<1.0	---	---
TSB (312 K.U.)	None	<1.0	<1.0	<1.0	1.0
	Buffer only	<1.0	<1.0	---	---
	+0.1% glucose	<1.0	<1.0	---	---
	+1.0% glucose	<1.0	<1.0	---	---
	+5.0% glucose	<1.0	<1.0	---	---
(B) VFC + 2μg niacin + 2μg thiamine (296 K.U.)	None	<1.0	<1.0	---	---
	PBG	76.4	4.6	---	---
	PBG+arg,cys,gln	1.7	2.7	---	---
	PBG+0.05 M NH_4H2PO_4	55.3	4.3	---	---
	PBG+0.5 M $NH_4H_2PO_4$	55.1	4.0	---	---
VFC + 2μg niacin (84 K.U.)	None	<1.0	<1.0	---	---
	PBG	3.0	<1.0	---	---
	PBG+arg,cys,gln	1.1	1.7	---	---

been involved.

3. Phosphate

To determine the effects of the presence of inorganic orthophosphate (Pi) in the growth medium, as well as the effects of increasing Pi concentration during incubation, S. aureus was grown in VFC and VFCA supplemented with niacin and thiamine, prepared in water or in 0.05 M K-Pi buffer, pH 7.0. To test the effects of increasing Pi concentration, cells were incubated in a non-Pi buffer (0.05 M tris-maleate, pH 7.0) with Pi added over a 100-fold concentration range. The results (Table 12) suggested a definite effect of Pi on the accumulation of FDP, both as a result of growth in the presence of Pi and of increasing concentrations during incubation. In part A, the addition of Pi to VFC and to VFCA resulted in inhibitions of FDP accumulation by 45% and 28%, respectively.

Growth in the presence of Pi caused inhibitions of 72%, 70%, and 60% in the accumulations of FDP after incubation with 0.005, 0.05, and 0.5 M Pi, respectively (Table 12, part B). However, after growth with Pi and incubation without Pi, FDP increased to levels higher than those in the cells grown and incubated without Pi. Growth with Pi also inhibited the accumulation of ATP by 80%, 77%, and 76% after incubation in the presence of 0.005 M, 0.05 M, and 0.5 M Pi, respectively.

4. Glucose and/or phosphate

Although it was shown previously that growth of S. aureus with added glucose did not significantly affect the zero-time levels of FDP or the other EM pathway intermediates, this did not eliminate the possibility that such growth would affect the accumulation of FDP during

Table 12. Effects of phosphate during growth and incubation on levels of intermediates in S. aureus[a]

Growth Medium	Klett Units	Incub. Medium	Intermediates, μmol/g G6P	FDP	DHAP	NAD	ATP
(A) VFC	174	None	---	<1.0	<1.0	1.1	---
		PBG	---	113.0	4.9	<1.0	---
VFC + Pi	389	None	---	<1.0	<1.0	4.0	---
		PBG	---	61.7	3.4	3.0	---
VFCA	225	None	---	<1.0	<1.0	4.3	---
		PBG	---	167.2	4.5	3.2	---
VFCA+Pi	276	None	---	<1.0	<1.0	5.6	---
		PBG	---	121.2	3.6	4.3	---
(B) VFC	182	None	<1.0	<1.0	<1.0	1.4	7.1
		TMG	<1.0	<1.0	<1.0	—	4.1
		+0.005MPi	<1.0	58.6	5.3	<1.0	25.3
		+0.05MPi	1.3	51.7	4.5	—	26.7
		+0.5MPi	1.8	20.6	2.3	—	18.1
VFC+Pi	385	None	<1.0	<1.0	<1.0	4.7	8.4
		TMG	<1.0	14.1	3.6	—	16.6
		+0.005MPi	<1.0	16.7	5.0	—	12.1
		+0.05MPi	<1.0	15.6	2.7	—	12.9
		+0.5MPi	<1.0	8.2	3.1	—	11.0

[a] S. aureus was grown in 2% VFC or 2% VFCA plus 2 μg each of niacin and thiamine, with or without 0.05 M K-Pi buffer, pH 7.0, as indicated, for 17 h. The cells were washed once in 0.8% NaCl (part A) or 0.8% KCl (part B), resuspended in the respective saline solutions, and added to the indicated incubation media. At zero time ("None") and at 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. The dry wt per incubation flask was 0.06–0.13 g in part A and 0.04–0.06 g in part B. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. TMG, 0.05 M tris-maleate buffer, pH 7.0 plus 1% glucose. In part B, Pi was added as the mono-and dipotassium salts, balanced for pH 7.0. (Two experiments.)

subsequent incubation. Accordingly, S. aureus was grown in VFCA plus glucose and/or Pi and incubated in PBG. The results (Table 13) showed effects of both glucose and Pi on pathways and intermediates. Growth with Pi stimulated the HMP pathway and the TCA cycle, but growth with glucose inhibited the TCA cycle.

The growth conditions had marked effects on the subsequent accumulation of FDP. Growth with Pi caused only a 6% inhibition of the accumulation of FDP, but growth with glucose caused a 78% inhibition. A 58% inhibition resulted when the cells were grown with both Pi and glucose.

5. NaCl

The halotolerant nature of staphylococci suggested that possible effects of growth in the presence of salt on the accumulation of FDP be tested. Cells were grown in VFC with or without 2% NaCl, then incubated in PBG with or without NaCl. Arsenate and arsenite were also included during incubation to test the effects of these inhibitors on the accumulation of FDP. The results of this experiment are shown as Table 14. In general, growth of S. aureus in the presence of salt resulted in lower overall levels of intermediates. Longer exposure to NaCl resulted in progressively smaller accumulations of FDP.

6. Cysteine

Cysteine was tested because it is an essential amino acid for staphylococci, and because of the effects of cysteine observed during incubation (Section II, B, 6 and 7). Two approaches were used. Firstly, the standard growth medium, VFCA, was supplemented with 1 or 5 mM cysteine-HCl. Secondly, two commercially-produced media were chosen which were similar in composition and general properties, but which differed in

Tables 13. Effects of growth with glucose and/or phosphate on levels of intermediates in non-growing S. aureus

Addition to Growth Medium	Klett Units	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM	μmol FDP/g	μmol DHAP/g
None	270	0	20.9	79.1	2.2	7.3	1.5	5.8	<1.0	<1.0
		3	14.3	85.7	1.3	6.7	1.0	5.7	93.4	4.7
Phosphate	349	0	30.8	69.2	5.7	7.4	2.3	5.1	<1.0	<1.0
		3	16.7	83.3	3.5	7.0	1.2	5.8	87.4	4.6
Glucose	232	0	19.0	81.0	0.4	6.7	1.3	5.4	<1.0	<1.0
		3	12.5	87.5	0.2	6.8	0.8	6.0	20.3	<1.0
Phosphate and glucose	482	0	15.3	84.7	0.7	7.4	1.1	6.3	<1.0	<1.0
		3	9.3	90.7	0.5	7.1	0.7	6.4	39.4	1.6

S. aureus was grown in 2% VFCA plus 2 μg niacin and thiamine per ml, plus the indicated additions, for 17 h, washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. Pi was supplied by preparing the media in 0.05 M K-Pi buffer, pH 7.0. After the medium was autoclaved, glucose was added aseptically to a final concentration of 1%. At 0 and 3 h of incubation, pathways were estimated and phenol extracts were prepared for spectrophotometric assay of intermediates. The cell density during incubation was 0.08-0.13 g dry wt per 100 ml in 300 ml total. (One experiment.)

Table 14. Effects of NaCl during growth and incubation, and effects of arsenate and arsenite during incubation, on levels of intermediates in S. aureus

Growth Medium	Incubation Medium	Incub. Time, h	Intermediates, μmol/g FDP	DHAP	PEP	Pyruvate	ATP
VFC	None	0	<1.0	<1.0	1.3	<1.0	19.1
	PBG	3	85.8	3.6	<1.0	<1.0	25.8
	PBG+NaCl	3	57.2	2.8	<1.0	1.0	25.1
	PBG+arsenate	3	7.8	<1.0	<1.0	2.4	29.4
	PBG+arsenite	3	2.6	<1.0	<1.0	6.5	28.8
VFC+NaCl	None	0	<1.0	<1.0	1.6	<1.0	18.1
	PBG	3	48.9	3.1	<1.0	1.1	24.6
	PBG+NaCl	3	33.0	2.3	<1.0	1.1	28.3
	PBG+arsenate	3	<1.0	<1.0	<1.0	2.4	16.1
	PBG+arsenite	3	<1.0	<1.0	<1.0	3.9	14.2

S. aureus was grown in 2% VFC + 2 μg each of niacin and thiamine per ml, with (254 Klett units) and without (303 Klett units) 2% NaCl. After 17 h, the cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At zero time and after 3 h of incubation, phenol extracts were prepared for spectrophotometric assay of intermediates. PBG, 0.1 M K-Pi buffer, pH 7.0, plus 1% glucose. Final concentrations: NaCl, 2% or 0.34 M; Na arsenate, 6.4 mM; Na arsenite, 7.7 mM. Cell density during incubation, 0.08-0.13 g dry wt per 100 ml total volume. (One experiment.)

their cysteine concentrations. These were N-Z-Amine and Edamin, with reported cystine concentrations of 0.0 and 67.2 mg/g, respectively. Prepared as 2% solutions yielded final cystine concentrations of 0.0 and 5.6 mM, respectively. The results of these experiments are presented in Table 15. Growth of cells in VFCA supplemented with cysteine resulted in greater accumulations of FDP, 31% in part A and 77% in part B. In contrast, cells grown in Edamin accumulated 48% less FDP than the cells grown in N-Z-Amine.

7. Growth medium

The experiment in which cells grown in TSB accumulated essentially no FDP (Table 11) suggested that the growth medium could definitely influence the accumulation of FDP during subsequent incubation. This was further substantiated by growth of S. aureus in N-Z-Amine and in Edamin, which resulted in smaller accumulations of FDP (Table 15). During the latter parts of this investigation, it became necessary to change the standard growth medium from VFC to VFCA because the former was no longer available. These media are similar, except that VFC was an enzymatic hydrolysate and VFCA an acid hydrolysate. As the use of new batches of VFCA was initiated, differences in the appearance of the medium and in the amount of growth of the organisms was observed. In a test of four new batches of VFCA, growth of the organisms differed considerably, but FDP accumulated to even higher levels than had been previously observed. The media were prepared as usual (2% VFCA supplemented with 2 μg each of niacin and thiamine per ml) and after 17 h of growth, the cells were incubated for 2 h in PBG. The level of FDP after incubation and the growth of the organisms in one experiment were: 137.8 μmol/g (245

Table 15. Effect of cysteine concentration during growth on the accumulation of FDP during incubation of S. aureus

Growth Medium	Klett Units	Incubation Medium	μmol FDP/g	μmol NAD/g
(A) VFCA	210	None	< 1.0	8.8
		PBG	92.4	5.2
VFCA+1 mM cys−HCl	260	None	< 1.0	7.2
		PBG	120.6	4.6
(B) VFCA	254	None	< 1.0	4.0
		PBG	60.4	2.4
VFCA+5 mM cys−HCl	262	None	< 1.0	5.0
		PBG	106.8	3.0
(C) N-Z-Amine (Type AS)	309	None	< 1.0	5.4
		PBG	69.2	3.2
		PBG-CYS−HCl	1.9	2.1
Edamin	302	None	< 1.0	5.2
		PBG	36.3	3.6
		PBG+CYS−HCl	4.0	2.5

Growth Medium	Incub. Medium	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM
(A) VFCA	None	24.1	75.9	2.6	7.4	1.8	5.6
	PBG	21.7	78.3	1.9	6.1	1.3	4.8
VFCA + 1 mM cys	None	28.9	71.1	3.9	6.3	1.8	4.5
	PBG	23.5	76.5	3.6	6.0	1.4	4.6

S. aureus was grown in the indicated media supplemented with 2 μg each of niacin and thiamine per ml for 17 h, washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated. Phenol extracts of the cells were prepared at 0 and 3 h of incubation for spectrophotometric assay of intermediates. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. Cysteine-HCl during incubation, 0.1 M, neutralized to pH 7.0 with KOH. N-Z-Amine and Edamin have low and high cysteine content, respectively, but otherwise are similar in composition. Cell density per incubation flask was 0.03-0.07 g dry wt, except in part A, cells grown with cysteine, 0.12 g dry wt, in a total volume of 100 ml per flask. For part (A), pathways were estimated at zero time ("None") and after 3 h of incubation. (Three experiments.)

Klett units), 147.3 μmol/g (71 Klett units), 121.6 μmol/g (88 Klett units), and 108.4 μmol/g (105 Klett units). (The cell density during incubation was 0.05-0.06 g dry wt per 100 ml total volume.)

The last medium tested was a synthetic medium (134). The results (Table 16) were generally comparable to those obtained with VFC. After incubation of the cells in PBG, they did not differ significantly in pathway percentages or in μmol of glucose utilized. The accumulations of FDP and of ATP, as well as the decrease in NAD levels, were all comparable to those observed in cells grown in VFC.

Table 17 summarizes the effect of the growth medium on the subsequent accumulation of FDP. Based on these results, the growth media may be arranged in the order of the extent of subsequent accumulation of FDP after incubation in PBG, from lowest to highest: TSB, Edamin, N-Z-Amine, VFC and synthetic medium, and VFCA. Because five of these media are undefined, precise reasons for the differences in FDP accumulation are not available.

8. Fresh growth medium

The standard procedure in this investigation was to harvest S. aureus after 17 h of growth, when the cells were in late log or early stationary phase. It was thus of interest to determine the effects of placing these cells into fresh growth medium compared to the usual incubation under non-growing conditions. Table 18 presents the results of several such experiments. Each control (PBG) showed substantial increases in the level of FDP and decreases in NAD. In the cells placed into fresh growth medium, the level of FDP increased insignificantly in the absence of glucose (parts A-C). The presence of glucose in the

Table 16. Effects of growth in synthetic medium on pathways and intermediates during incubation of S. aureus

	Incubation Medium	Incub. Time, h	% HMP	% EM	TCA ACT.	% glucose used	Glucose used, µmol per h	Glucose used, µmol via HMP	Glucose used, µmol via EM	Intermediates, µmol/g G6P	Intermediates, µmol/g FDP	Intermediates, µmol/g DHAP	Intermediates, µmol/g NAD	Intermediates, µmol/g ATP
(A)	None	0	23.1	76.9	1.0	66.4	7.9	1.8	6.1	<1.0	<1.0	<1.0	4.5	9.0
	PBG	2	12.7	87.3	1.1	54.5	6.6	0.8	5.8	3.5	100.2	3.6	2.5	28.4
	PBG+100mM cys-HCl	2	22.4	77.6	0.4	39.7	4.9	1.1	3.8	<1.0	3.8	1.6	1.4	16.4
(B)	None	0(a)	21.1	78.9	1.4	76.5	9.3	2.0	7.3	<1.0	<1.0	<1.0	5.8	9.9
		(b)	20.3	79.7	0.3	69.2	8.3	1.7	6.6	–	–	–	–	–
	PBG	2(c)	24.1	75.9	4.8	61.0	7.4	1.8	5.6	1.2	78.2	3.6	3.9	26.3
	PBG+100mM cys-FB	2(d)	24.7	75.3	0.2	52.2	6.0	1.5	4.5	<1.0	11.8	2.1	2.5	18.2
	PBG+100mM cys-HCl		----	----	---	----	---	---	---	<1.0	4.8	1.6	2.7	18.5

S. aureus was grown in the synthetic medium of Idriss and Blumenthal (134) for 17 h (part A, 226 Klett units; part B, 292 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0 and incubated. Pathways were estimated and phenol extracts were prepared at 0 and 2 h. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. Cys-HCl, cysteine hydrochloride; cys-FB, cysteine free base. Cell density was 0.06-0.08 g dry wt per 100 ml total volume. Note, in part B: (a) and (c), pathways estimated as described in Methods; (b) and (d), pathways estimated in the presence of 100 mM cysteine free base, added directly to the estimation flask. All media were adjusted to pH 7.0 after addition of cysteine. (Two experiments.)

Table 17. Effect of growth medium on accumulation of FDP in S. aureus during incubation in phosphate-buffered glucose

Growth Medium	Growth[a] Klett Units	Incubation time, h	μmol FDP/g
2% Vitamin-Free Casitone	241 (8) [171-329]	0	<1.0
		3	87.3 ± 17.3 (14)[b]
2% Vitamin-Free Casamino Acids	240 (10) [210-273]	0	<1.0
		3	116.1 ± 38.6 (10)[b]
3% Trypticase Soy Broth	312 (2)	0	<1.0
		2.5	<1.0
2% N-Z-Amine Type AS	309 (1)	0	<1.0
		3	69.2
2% Edamin	302 (1)	0	<1.0
		3	36.3
Synthetic medium	259 (2) [220-315]	0	<1.0
		2	89.2[c]

S. aureus was grown in the indicated media supplemented with 2 μg each of niacin and thiamine per ml (except Trypticase Soy Broth, which was unsupplemented) for 17 h, washed once in 0.05 M K-Pi buffer, pH 7, and incubated in 0.05 M K-Pi buffer, pH 7, plus 1% glucose. At the indicated times, phenol extracts were prepared for assay of FDP as described in Methods.

[a] The value is the mean of the number of experiments indicated in parenthesis; the range of values is shown in brackets.

[b] The value is the mean ± the standard deviation for the number of experiments shown in parenthesis.

[c] Mean of 78.2 and 100.2.

Table 18. Effect of fresh growth medium on levels of intermediates in S. aureus

Incubation Medium		Incub. Time, h	Intermediates, μmol/g FDP	DHAP	NAD
(A)	None	0	<1.0	<1.0	3.0
	PBG	3	92.8	2.8	2.6
	PBG+2500 μg 6AN/ml	3	68.1	2.4	2.2
	2% VFC+vitamins	3	1.0	<1.0	3.8
(B)	None	0	<1.0	<1.0	3.3
	PBG	3	102.1	4.6	2.6
	PBG+2500 μg 6AN/ml	3	99.5	3.9	1.6
	2% VFC+vitamins	3	<1.0	<1.0	4.6
	2% VFC+vitamins + 1% glucose	3	2.2	<1.0	6.7
(C)	None	0	<1.0	<1.0	4.2
	PBG	3	66.3	4.0	2.8
	2% VFC+vitamins	3	1.1	<1.0	5.0
	2% VFC+vitamins + 1% glucose	3	1.5	<1.0	7.3
(D)	None	0	<1.0	<1.0	4.1
	2% VFC+vitamins	3 1/2	<1.0	<1.0	4.1

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (part A, 197 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. Phenol extracts for spectrophotometric assay of intermediates were prepared at zero time and at the end of incubation. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. 6AN, 6-aminonicotinamide. Niacin and thiamine, 2 μg/ml. Cell densities were: Part A, 0.13-0.15 g dry wt; part B and C, 0.10-0.14 g dry wt, in 100 ml total volume. For part D, after 17 h of growth, the cells were resuspended to the same cell density, 0.41 g dry wt in 300 ml. (4 experiments.)

fresh growth medium caused only very slight increases in FDP (parts B and C).

In part D, in which the cells were resuspended to the same density that they had attained after 17 h of growth, the level of FDP did not change significantly and NAD remained constant.

These results indicated that FDP levels changed insignificantly when cells were placed into fresh growth medium, but with glucose there was a very slight increase. These changes may have been related to the conditions caused by the renewal of growth in the fresh medium.

B. Factors affecting FDP levels during incubation of non-growing cells

The second phase of this investigation, as mentioned earlier, was devoted to an examination of some of the factors involved in the accumulation of FDP. Because FDP concentrations were always low in growing cells, because FDP never accumulated in growing cells, and because the factors presented in the previous section were present during growth but only affected FDP levels in the non-growing cells, a search was initiated for the factor or factors that would significantly reduce, or even prevent, the accumulation of FDP in the non-growing cells.

1. Washing of cells

As mentioned previously, washing of the cells after incubation was found to have a significant effect on the observed level of FDP. The data in Table 7 showed that three washes reduced the observed levels of FDP and 6PG by as much as 49% and 86%, respectively. Although this was only a technical problem, it resulted in the standardization of the post-incubation washing procedure.

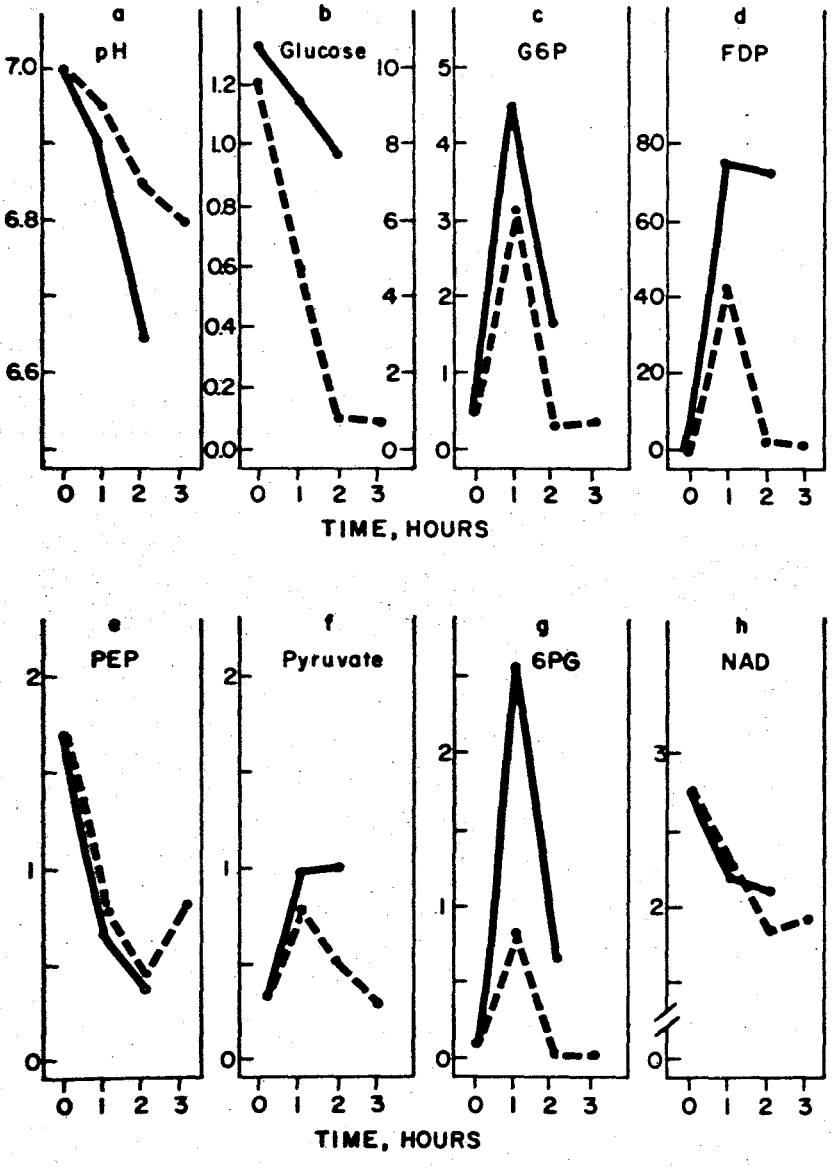
2. Glucose concentration and the spontaneous reversal of FDP accumulation

Because FDP was metabolically derived from glucose during incubation, it seemed reasonable to examine the effect of the glucose concentration during incubation on the accumulation of FDP. Two approaches were used. In the first, cells were grown under several different conditions and incubated in phosphate buffer with glucose concentrations of 0, 0.1, 1.0, and 5.0%. The results (Table 11, part A) showed that after 2 h of incubation, the accumulation of FDP increased with increasing glucose concentration.

The second approach was a comparison of two glucose concentrations during incubation, the usual 1% (55.6 mM), and the concentration of glucose used for estimation of the pathways, 1.125 mg/ml or 6.25 mM. This latter concentration is 1/9 the concentration of 1% glucose (10 mg/ml). The results are presented as Figure 2. As might be expected, the pH of the incubation medium decreased more rapidly in the presence of 1% glucose. The cells incubated in 1% glucose used 218 mg or 26% of the total available after 2 h, whereas the cells incubated in 0.1% glucose used a smaller amount (95 mg) but a greater proportion (95%) of the glucose available. The intermediates G6P, FDP, and 6PG increased in the presence of 1% glucose, and G6P and 6PG actually decreased after 2 h of incubation. FDP gave some evidence of decreasing by 2 h. In the cells incubated in 0.1% glucose, the same intermediates increased after 1 h of incubation, and by 3 h had decreased to as low or lower than the zero time levels. (Unfortunately, a 3 h extraction of cells incubated in 1% glucose was not prepared.) Similar to previous experiments, PEP and

Figure 2. S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h, washed once in 0.1 M K-Pi buffer, pH 7.0, and adjusted to a cell density of 1800 Klett units (using a 1:10 dilution reading 180 Klett units). The purpose of this was to incubate cells under conditions that resembled those used for the pathways estimation. In this 20-fold scaled-up experiment, 50 ml of cell suspension was added to 250 ml Erlenmeyer flasks containing 20 ml 0.1 M K-Pi buffer, pH 7.0, and 10 ml of glucose solution, 9 mg/ml (0.05 M), or 8%. The final concentrations of glucose were thus 1.125 mg/ml (6.25 mM), equivalent to that used in the pathways estimation, and 1%, as used in the standard incubation experiments. The cell density in this experiment was 0.16 g dry wt per 80 ml incubation medium, equivalent to 0.2 g dry wt/100 ml, or 2-3 fold higher than used in the standard incubation experiments. At 0, 1, 2, and 3 h of incubation, phenol extracts were prepared for spectrophotometric assay of intermediates. Glucose was assayed using the anthrone test.

Fig. 2. Effect of incubation of S. aureus in two concentrations of glucose on pH of the medium, glucose utilization, and metabolic intermediates.



NAD decreased in cells at both glucose concentrations.

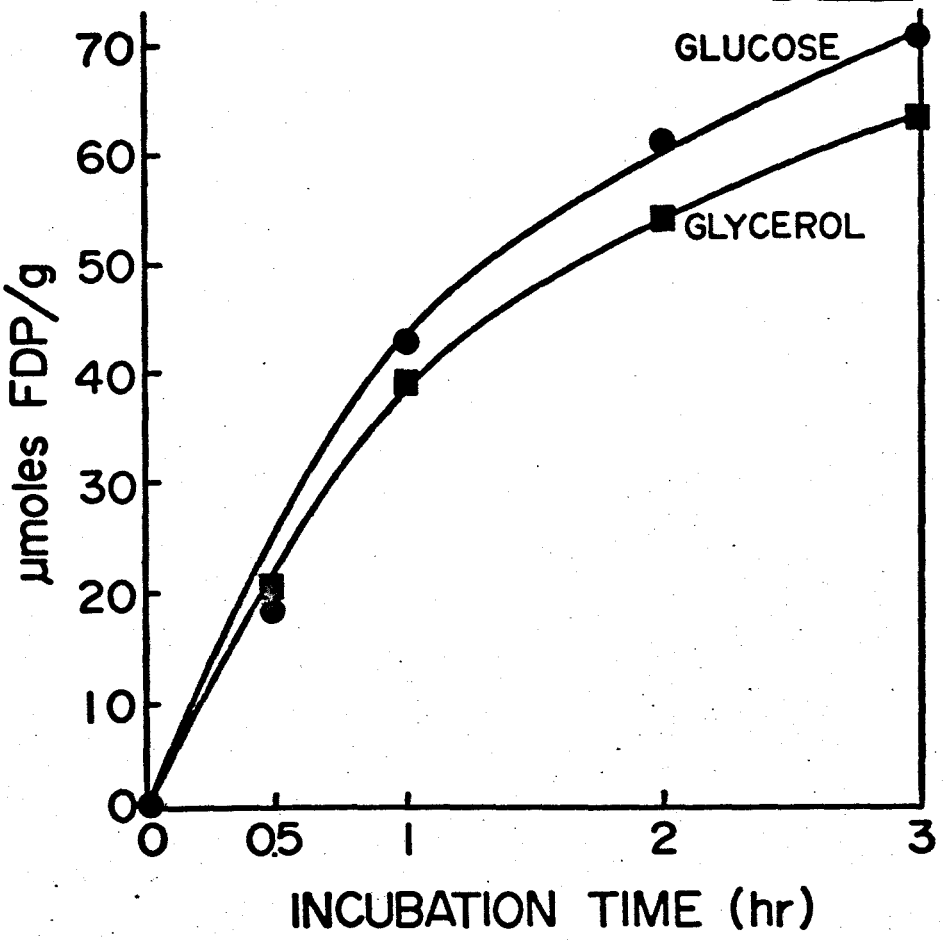
These two experiments emphasize the direct relationship between the EM intermediates and glucose. As shown previously (Tables 5, 8, and 9), no EM intermediates accumulated in the absence of glucose. The experiment of Table 11, part A showed that the extent of FDP accumulation increased with increasing glucose concentration in the incubation medium. The experiment of Figure 2 re-emphasized the relationship between glucose concentration and accumulations of intermediates (G6P, 6PG, as well as FDP), and also showed that the accumulations would spontaneously reverse, apparently when the glucose was sufficiently depleted from the medium.

3. Carbohydrates other than glucose

Staphylococci are capable of utilization of a number of carbohydrates in addition to glucose, and the effect of a number of carbohydrates on the level of FDP during incubation was tested. Figure 3 shows the results of incubation of S. aureus in 0.05 M K-Pi buffer, pH 7.0, plus either glucose or glycerol at 1% final concentration. Over the 3 h incubation, glycerol was almost as effective as glucose in causing the accumulation of FDP. The results of several other experiments are presented as Table 19. The effectiveness of glycerol is again indicated in parts A and B, where it caused accumulations of G6P, F6P, and FDP comparable to those caused by glucose. Parts C and D indicate that galactose caused a substantial accumulation of FDP, and that mannose, fructose, and ribose were less effective. The smallest accumulation occurred in the presence of mannitol.

4. Phosphate and non-phosphate buffers

Figure 3. Effects of glucose and glycerol on levels of intermediates during incubation of S. aureus



S. aureus was grown in 2% VFC plus 2 µg each of niacin and thiamine per ml for 17 h (335 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus either 1% (w/v) glucose or 1% (v/v) glycerol. At the indicated times, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation was 0.10-0.12 g dry wt/100 ml in 300 ml total. (One experiment.)

Table 19. Effect of glucose and other carbohydrates on levels of intermediates during incubation of S. aureus

	Addition to 0.05M K-Pi Buffer, pH 7.0	Incub. Time, h	Intermediates, μmol/g G6P	F6P	FDP	DHAP	NAD	ATP
(A)	None	0	<1.0	<1.0	<1.0	<1.0	3.3	---
	Glucose	3	1.8	2.8	89.4	1.1	2.4	---
	Glycerol	3	1.1	2.5	97.2	<1.0	2.0	---
(B)	None	0	---	---	<1.0	<1.0	3.7	---
	Glucose	3	---	---	76.2	1.8	2.4	---
	Glycerol	3	---	---	86.0	2.4	2.3	---
(C)	None	0	---	---	<1.0	<1.0	1.1	---
	Glucose	3	---	---	70.3	4.1	<1.0	---
	Glycerol	3	---	---	35.8	2.9	<1.0	---
	Fructose	3	---	---	14.7	2.3	<1.0	---
	Galactose	3	---	---	47.0	3.2	<1.0	---
	Mannose	3	---	---	23.9	3.0	<1.0	---
(D)	None	0	<1.0	---	<1.0	<1.0	3.8	10.6
	Glucose	3	<1.0	---	73.0	3.5	2.7	28.7
	Mannitol	3	<1.0	---	6.0	1.2	3.5	19.5
	Ribose	3	<1.0	---	12.2	1.6	3.3	21.3

S. aureus was grown in 2% VFC plus 2 μg niacin and thiamine per ml for 17 h. Cell densities were: part A, 345 Klett units; part B, 334 Klett units; part D, 245 Klett units. The cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus the indicated carbohydrates at 1% final concentration. All carbohydrates were added as the solid (1% w/v), except glycerol (1% v/v). At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities during incubation were: parts A and B, 0.09-0.11 g dry wt/100 ml in 300 ml total; parts C and D, 0.06-0.09 g/100 ml and 0.10-0.15 g/100 ml, respectively, in 100 ml total.

Phosphate buffer was used routinely in this investigation, both for washing and incubation of cells. It was realized, however, that Pi was not only a buffer, but also was a potential metabolite. Because of the very large accumulations of FDP that were observed and because FDP is a diphosphorylated carbohydrate, it was reasonable to examine the role of Pi in this phenomenon. Incubation of S. aureus in non-Pi buffers (tris-maleate or triethanolamine) with Pi concentrations of 0, 0.005, 0.05, and 0.5 M (adjusted to pH 7.0) yielded the results shown in Table 12, part B, and in Table 20, part A. Maximum accumulations of FDP occurred at Pi concentrations of 0.005 or 0.05 M. The 10-fold increase of Pi, from 0.005 to 0.05 M, had no significant effects on PEP, pyruvate, or NAD. The effects of increasing Pi concentrations were also shown in the levels of ATP (Table 12, part B), which paralleled those of FDP. This occurred both in cells grown with and without Pi, but the accumulations of FDP and ATP were smaller in the cells grown with Pi than in the corresponding cells grown without Pi.

The role of Pi in the accumulation of FDP was emphasized by incubation of cells in non-Pi buffers without added Pi (Table 20, part B). The maximum accumulation of FDP in the non-Pi buffers was only 21% of the Pi control. Similarly, ATP increased by a maximum of only 24% in the non-Pi buffers compared to the control. Again, as shown in Table 12, part B, the levels of ATP paralleled those of FDP.

These results suggested a definite relationship between (1) inorganic phosphate and FDP and (2) inorganic phosphate and ATP. Other results, presented in later sections, served to substantiate these relationships.

Table 20. Effect of phosphate concentration and non-phosphate buffers on levels of intermediates during incubation of S. aureus

	Incubation Medium Containing 1% Glucose	Incub. Time, h	Intermediates, μmol/g FDP	DHAP	NAD	ATP
(A)	None	0	<1.0	<1.0	1.2	---
	0.05 M TEA buffer	3	<1.0	<1.0	<1.0	---
	+0.005 M Pi	3	66.0	3.9	<1.0	---
	+0.05 M Pi	3	72.2	3.9	<1.0	---
	+0.5 M Pi	3	33.4	2.9	<1.0	---
	0.05 M K-Pi	3	82.4	3.5	<1.0	---
	0.1 M K-Pi	3	66.3	3.2	<1.0	---
	0.05 M Na-Pi	3	71.3	5.6	<1.0	---
	0.1 M Na-Pi	3	75.3	6.9	<1.0	---
(B)	None	0	<1.0	<1.0	3.8	10.6
	0.05 M K-Pi buffer	3	73.0	3.5	2.7	28.7
	0.1 M HEPES buffer	3	15.4	2.1	2.0	12.9
	0.1 M TEA buffer	3	14.5	1.8	2.0	15.0
	0.1 M TM buffer	3	8.5	1.4	2.2	12.4

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (part A, 193 Klett units; part B, 245 Klett units). In part A, cells were washed once in 0.056 M TEA buffer, resuspended in the same buffer, and added to the indicated media. In part B, cells were washed once and resuspended in 0.8% KCl before addition to the indicated media. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities were: part A, 0.06-0.11 g dry wt; part B, 0.11-0.12 g dry wt/100 ml total volume. All buffers were pH 7.0. Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TEA, triethanolamine; TM, tris-maleate.

5. Arsenate, arsenite, and NaCl

The effects of arsenate and arsenite were tested because of their known inhibitory effects on the EM pathway. Arsenate may replace Pi and serve as an uncoupling agent (47). Arsenite inhibits the metabolism of alpha-keto acids (140). The results in Table 14 show the effects of incubation of S. aureus in the presence of 6.4 mM arsenate, 7.7 mM arsenite, & 2% (0.34M) NaCl, after growth with or without 2% NaCl. After growth without salt, incubations with arsenate and arsenite resulted in increases in FDP levels that were only 9% and 3% of the control, respectively. After growth with NaCl and incubation with arsenate and arsenite, the accumulations of FDP were insignificant. Arsenate and arsenite also had a considerable effect on the level of pyruvate during incubation.

The only observed effects of incubation of cells in the presence of NaCl were the lower accumulations of G6P and FDP. The increases in the level of FDP were 67% of the control, in the cells grown both with or without NaCl.

These results suggested that the metabolic reactions involving FDP, PEP, and pyruvate were sensitive to both arsenate and arsenite. NaCl exerted its effects both during growth and incubation of the cells, and the intensity of the effect on FDP was directly related to the length of exposure of the cells.

6. Amino acids

In order to establish the basis for the accumulation of FDP during incubation, a comparison was made which revealed the following: FDP did not accumulate in growing cells, even in the presence of glucose. FDP only accumulated in large amounts during incubation of S. aureus under

non-growing conditions in Pi buffer in the presence of glucose. Both glucose and Pi were essential for the accumulation of FDP (Tables 5,8,9, 12, and 20). Therefore, the only difference between growth and incubation conditions was the presence of amino acids during the growth of the cells. This suggested that addition of one or more amino acids to the incubation medium might inhibit the accumulation of FDP and eventually be of value in localizing a potential control point for FDP metabolism. Accordingly, a series of experiments was initiated which resulted in the finding that a single amino acid, cysteine, could prevent the accumulation of FDP during incubation.

In the initial experiments, commercially-prepared solutions of amino acids were used. These were Eagle's Minimum Essential Medium (without glutamine) and Non-Essential Amino Acids, provided by Grand Island Biological Company (Grand Island, NY) in 50- and 100-fold concentrated solutions, respectively. These were initially added to PBG at 2% and 4%, by volume, and some inhibition of the accumulation of FDP occurred (Table 21, part A). To emphasize the potential effects of the amino acid solutions, they were used at essentially full strength (95%, by volume). The essential amino acid solution (EAA) allowed an increase of FDP only 2-3% of the control (Table 21, parts B and C), whereas the non-essential amino acids (NEAA) were much less effective (Table 21, part B).

The thirteen amino acids in the EAA solution were then tested individually for their effects on the accumulation of FDP. The concentrations of the amino acids listed in Table 22 were the same as in the full strength commercial EAA solution, with the exception of isoleucine. The results of this survey (Table 22) indicated that arginine, cystine,

Table 21. Effect of amino acid mixtures on intermediates during incubation of S. aureus

Addition to 0.05 M K-Pi buffer, pH 7.0 plus 1% glucose	Incub. Time, h	Intermediates, μmol/g FDP	DHAP	NAD	FDP: % of Control
(A) None	0	<1.0	<1.0	3.0	--
None	3	92.8	2.8	2.6	100
2% (v/v) EAA	3	59.1	1.8	2.5	64
4% (v/v) EAA	3	45.6	1.3	2.2	49
2% (v/v) NEAA	3	75.1	1.3	2.4	81
4% (v/v) NEAA	3	71.4	1.0	2.5	77
4% (v/v) EAA+4% (v/v) NEAA	3	54.1	<1.0	1.8	58
(B) None	0	<1.0	<1.0	3.3	--
None	3	102.1	4.6	2.6	100
95% (v/v) EAA	3	2.0	<1.0	1.9	2
95% (v/v) NEAA	3	66.1	3.8	2.5	65
(C) None	0	<1.0	<1.0	4.2	--
None	3	66.3	4.0	2.8	100
95% (v/v) EAA	3	2.1	<1.0	2.7	3

S. aureus was grown in 2% VFC plus 2 μg of niacin and thiamine per ml for 17 h (part A, 197 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose, and the indicated additions. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation: part A, 0.13-0.16 g dry wt (except the last mixture, 0.23 g dry wt); parts B and C, 0.10-0.14 g dry wt, per 100 ml total volume. EAA, Eagle's Minimum Essential Medium (essential amino acids). NEAA, Eagle's Non-Essential Amino Acids.

Table 22. Effect of individual amino acids on levels of FDP and DHAP during incubation of S. aureus

	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	mM	Incub. Time, h	μmol FDP/g	μmol DHAP/g	FDP: % of Control
(A)	None	—	0	<1.0	<1.0	—
	None	—	3	109.0	5.6	100
	Arginine-HCl	25	3	56.0	3.4	51
	Cystine	5	3	58.4	4.5	54
	Leucine	20	3	71.5	4.3	66
	Lysine-HCl	16	3	101.5	5.4	93
	Phenylalanine	10	3	90.6	5.3	83
	Valine	20	3	94.2	6.2	86
	6 amino acids combined	—	3	42.6	4.8	39
(B)	None	—	0	<1.0	<1.0	—
	None	—	3.5	86.2	4.1	100
	Glutamine	100	3.5	32.9	2.2	38
	Histidine	10	3.5	67.5	2.3	78
	Isoleucine	40	3.5	61.1	4.2	71
	Methionine	5	3.5	84.9	3.4	98
	Threonine	20	3.5	123.0	2.1	143
	Tryptophan	2	3.5	72.0	3.1	84
	Tyrosine	10	3.5	88.3	1.2	102

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (part A, 268 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose, and the indicated additions. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation: part A, 0.06–0.09 g dry wt; part B, 0.11–0.15 g dry wt per 100 ml total. All amino acids were of the L configuration, except isoleucine, which was DL, hence used at twice the concentration in the commercial amino acid solution. (Two experiments.)

leucine, and glutamine were the most effective in inhibiting the accumulation of FDP during incubation.

As a check on the effectiveness of the 95% EAA solutions during incubation, the 13 separate amino acids were combined (at the same concentration) during incubation, and the results (Table 23, parts A and B) were comparable to those with the 95% commercial solutions (Table 21, parts B and C).

Various combinations of arginine, cysteine, glutamine, and leucine were then tested. (Cysteine replaced cystine because of the greater solubility of the former.) The concentrations used were the same as in the full strength commercial solution with the exception of cysteine. The commercial solution contained 1200 mg cystine/l. To obtain the same concentration of equivalents of cysteine, this was doubled to 2400 mg cysteine/l, which happened to equal a 15 mM solution of cysteine-HCl. The data in Table 23, part B indicated that doubling the concentration of arginine, cysteine, glutamine, and leucine from 25, 15, 100, and 20 mM, respectively, to 50, 30, 200 and 40 mM had a greater effect in inhibiting the accumulation of FDP. Elimination of leucine at the higher concentration was even more effective, but elimination of cysteine was much less effective. Table 23, part C again shows the effect of doubling the concentration of arginine, cysteine, and glutamine on FDP, and part D shows that glutamic acid and glutamine were equally effective in combination with the other two amino acids.

This series of experiments suggested that the accumulation of FDP during incubation could be greatly reduced by the presence of three amino acids in the incubation medium. The next section will present data concerning the effects of cysteine alone on FDP levels.

Table 23. Effect of amino acid combinations on levels of intermediates during incubation of S. aureus

	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	Intermediates, μmol/g FDP	DHAP	ATP
(A)	None	0	<1.0	<1.0	---
	None	3.5	86.2(100)	4.1	---
	13 amino acids combined	3.5	3.2(4)	<1.0	---
(B)	None	0	<1.0	<1.0	---
	None	3	59.1(100)	3.0	---
	13 amino acids combined	3	2.0(3)	<1.0	---
	Arg(25mM), cys(15mM), gln(100mM), leu(20mM)	3	10.3(17)	2.2	---
	Arg(50mM), cys(30mM), gln(200mM), leu(40mM)	3	6.7(11)	2.3	---
	Arg(50mM), cys(30mM), gln(200mM)	3	3.5(6)	2.8	---
	Arg(50mM), cys(30mM), leu(40mM)	3	7.0(12)	3.0	---
	Arg(50mM), gln(200mM), leu(40mM)	3	28.1(47)	1.6	---
(C)	None	0	<1.0	<1.0	---
	None	3	70.3(100)	4.1	---
	Arg(25mM), cys(15mM), glu(100mM)	3	13.7(19)	4.0	---
	Arg(50mM), cys(30mM), glu(200mM)	3	8.0(11)	3.2	---
(D)	None	0	<1.0	<1.0	10.6
	None	3	73.0(100)	3.5	28.7
	Arg(50mM), cys(30mM), glu(200mM)	3	3.0(4)	1.4	9.3
	Arg(50mM), cys(30mM), gln(200mM)	3	3.3(4)	1.4	9.1

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (part B, 271 Klett units; part D, 245 Klett units), washed once and resuspended in 0.05 M K-Pi buffer, pH 7.0 (except for cells in part D, which were washed once and resuspended in 0.8% KCl). The cells were incubated in 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose, plus the indicated additions. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities during incubation: part A, 0.13–0.15 g dry wt/100 ml; part B, 0.10–0.13 g/100 ml, except the last incubation mixture, 0.20 g/100 ml; part C, 0.06–0.09 g/100 ml; part D, 0.12–0.13 g/100 ml. The numbers in parentheses under μmol FDP/g indicate the % increase relative to the control (100%). Arginine-HCl and cysteine-HCl were used, and the incubation medium was readjusted to pH 7.0.

7. Cysteine

The next series of experiments demonstrated that cysteine alone could prevent the accumulation of FDP during incubation of S. aureus. Table 24 presents data concerning the effects of the amino acids, as well as of cysteine alone, on the pathways and on levels of intermediates. Incubation of cells in the presence of arginine, cysteine, and glutamine had almost no effect on either the percentage or the amount of glucose used by the pathways, although the activity of the TCA cycle was reduced (Table 24, part A). The accumulation of FDP with the three amino acids present was only 5% of the control. Cysteine, at 100 mM during incubation, greatly reduced the activity of the TCA cycle (Table 24, part B). The accumulation of FDP in the presence of 100 mM cysteine was insignificant compared to the control.

Incubation with arginine and glutamine, with or without cysteine, had little effect on the percent HMP pathway or on the utilization of glucose (Table 24, part C). Cysteine alone inhibited the EMP pathway, both in percentage and amount of glucose used, and inhibited the TCA cycle, both when cysteine was present alone or with arginine and glutamine. The accumulation of FDP in the presence of arginine, cysteine, and glutamine was only 8% of the control. Cysteine alone allowed an accumulation of FDP that was 15% of the control, and arginine and glutamine allowed a 44% accumulation.

Incubation of S. aureus in PBG plus cysteine at 60 mM greatly inhibited both the percentage and the amount of glucose utilized via the HMP pathway, and inhibited the TCA cycle (Table 24, part D). The accumulation of FDP in the presence of cysteine was only 4% of the control. The presence of 50 mM pyruvate during incubation did not affect the

Table 24. Effects of amino acids on pathways and intermediates during incubation of S. aureus

Expt.	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM	μmol FDP/g	μmol NAD/g
(A)	None	0	16.2	83.8	1.8	8.6	1.4	7.2	<1.0	6.4
	None	3	15.2	84.8	2.0	7.7	1.2	6.5	71.6	3.9
	Arg(50mM), cys(30mM), gln(200mM)	3	15.7	84.3	0.3	7.3	1.2	6.1	4.0	1.2
(B)	None	0	27.6	72.4	3.7	8.2	2.3	5.9	<1.0	7.0
	None	3	24.0	76.0	2.9	6.0	1.4	4.6	151.0	5.2
	Cys(100mM)	3	1.5	98.5	0.1	2.8	T	2.8	<1.0	2.1
	Iodoacetate(1mM)	3	1.4	98.6	0.4	4.2	0.1	4.1	84.6	4.7
(C)	None	0	25.0	75.0	3.0	7.9	2.0	5.9	<1.0	8.1
	None	3	18.5	81.5	1.9	7.5	1.4	6.1	108.9	4.3
	Arg(50mM), cys(30mM), gln(200mM)	3	18.9	81.1	0.4	6.6	1.2	5.4	8.5	3.9
	Cys(30mM)	3	10.8	89.2	0.2	3.8	0.4	3.4	15.9	2.1
	Arg(50mM), gln(200mM)	3	16.6	83.4	1.5	6.8	1.1	5.7	44.1	4.5
(D)	None	0	22.2	77.8	3.7	7.8	1.7	6.1	<1.0	8.3
	None	3	17.9	82.1	2.0	6.8	1.2	5.6	126.6	5.8
	Cys(60mM)	3	1.5	98.5	0.1	4.0	0.1	3.9	5.0	1.9
	Cys(60mM), niacin (100 μg/ml)	3	1.7	98.3	0.04	4.5	0.1	4.4	5.8	1.7
	Pyruvate(50mM)	3	17.4	82.6	1.7	5.8	1.0	4.8	102.9	4.6

S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h. Cell densities after 17 h of growth: part A, 212 Klett units; part B, 196; part C, 232; part D, 262 Klett units. The cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At 0 and 3 h, pathways were estimated and phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities during incubation: part A, 0.10-0.14 g dry wt; part B, 0.10 g; part C, 0.06-0.09 g; part D, 0.10-0.12 g/100 ml total volume. These values are somewhat low because some cells (30-50%) were removed for use in the pathways estimation. The HCl salts of arginine and cysteine were used.

percentage of the pathways, but caused similar slight reductions in the amount of glucose utilized and allowed FDP to accumulate to 81% of the control.

Cysteine thus appeared to exert inhibitory effects on both the percentage and the amount of glucose metabolized via the HMP pathway, as well as inhibiting the TCA cycle, compared to arginine and glutamine. Cysteine alone almost completely inhibited the accumulation of FDP, and accelerated the decrease in NAD levels.

The effects of cysteine on the HMP pathway and on FDP were emphasized by incubation of S. aureus in PBG with increasing concentrations of cysteine-HCl. Table 25, part A shows that cysteine at 1 or 5 mM had very little effect on the HMP pathway, but allowed increases of FDP by 35% and 46%, respectively. The decreases in NAD were 46% and 72%, respectively, compared to 31% in the control. Cysteine at 30 mM allowed an accumulation of FDP that was only 5% of the control, and the decrease in NAD was 90%, compared to 35% in the control (Table 25, part B). The accumulation of FDP with 10 mM cysteine was 23% of the control, in Table 25, part A, and 18%, part C. Table 25, part C also shows the effects of cysteine at 30, 60, and 100 mM. The % HMP pathway was inversely related to the cysteine concentration, decreasing from 2.3% HMP at 10 mM cysteine to 0.5% at 100 mM cysteine. The amount of glucose utilized under these conditions was insignificant. The activity of the TCA cycle was also greatly reduced. Glucose utilization overall was reduced in the presence of cysteine. The accumulation of FDP was reduced as the concentration increased. At 10, 30, 60, and 100 mM cysteine, the accumulations of FDP were only 18%, 3%, 0.8%, and 0.2% of the control, respectively. The

Table 25. Effects of cysteine on pathways, FDP, and NAD during incubation of S. aureus

Expt.	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	via HMP	via EM	Intermediates, μmol/g FDP	DHAP	NAD
(A)	None	0	19.6	80.4	2.3	8.5	1.7	6.8	<1.0	<1.0	6.8
	None	3	15.6	84.4	1.8	6.4	1.0	5.4	177.7	5.3	4.7
	CYS (1mM)	3	14.5	85.5	0.5	5.8	0.8	5.0	62.3	4.3	3.7
	CYS (5mM)	3	18.1	81.9	0.4	5.8	1.0	4.8	81.6	4.3	1.9
	CYS (10mM)	3	12.6	87.4	0.2	4.8	0.6	4.2	41.6	3.2	1.6
(B)	None	0	---	---	---	---	---	---	<1.0	<1.0	6.8
	None	3	---	---	---	---	---	---	126.6	1.8	4.4
	CYS (30mM)	3	---	---	---	---	---	---	6.0	1.3	<1.0
(C)	None	0	20.6	79.4	2.0	8.3	1.7	6.6	<1.0	<1.0	7.2
	None	3	14.5	85.5	1.1	6.6	1.0	5.6	137.9	4.1	4.7
	CYS (10mM)	3	2.3	97.7	0.1	3.2	0.1	3.1	24.7	3.3	<1.0
	CYS (30mM)	3	1.2	98.8	0.04	3.8	T	3.8	4.3	2.4	<1.0
	CYS (60mM)	3	1.1	98.9	0.04	4.0	T	4.0	1.2	1.8	1.0
	CYS (100mM)	3	0.5	99.5	0.03	4.2	T	4.2	<1.0	1.7	1.5

S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (Klett units: part A, 221; part B, 273; part C, 252), washed once in 0.05 M K-Pi buffer, and incubated in the indicated media. At 0 and 3 h, pathways were estimated and phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation was 0.06-0.08 g dry wt per 100 ml total volume. Cysteine-HCl was dissolved in the buffer, which was readjusted to pH 7.0.

respective decreases in NAD were 90%, 89%, 86%, and 79%, compared to 35% in the control.

Increasing concentrations of cysteine appeared to greatly reduce both the percentage and amount of glucose catabolized by the HMP pathway, as well as reducing total glucose utilization and the activity of the TCA cycle. The accumulation of FDP was greatly reduced as the cysteine concentration increased, with 100 mM cysteine causing almost complete inhibition.

The effects of incubation of S. aureus in 0.05 M K-Pi buffer, pH 7.0, with or without 1% glucose and/or 100 mM cysteine are shown as Table 26. The addition of cysteine to PBG reduced both the percent and the amount of glucose used via the HMP pathway, and the addition of cysteine to the buffer caused a greater reduction. Cysteine caused a large reduction in the activity of the TCA cycle, both in the presence and absence of glucose. Cysteine also caused a reduction in total glucose utilization. The accumulation of FDP in PBG plus cysteine was only 0.4% of the control, and the decrease in the level of NAD was 48%, compared to 25% in the control. As expected, no FDP accumulated in cells incubating in buffer only. The decrease in NAD in the cells incubating in buffer with or without cysteine was 25% and 2%, respectively.

This series of experiments indicated that incubation of S. aureus in PBG plus cysteine resulted in: (1) a decrease in the percent of glucose catabolized by the HMP pathway; (2) a decrease in the amount of glucose utilized via the HMP pathway; (3) a decrease in the total amount of glucose utilized; (4) a decrease in the activity of the TCA cycle; (5) an inhibition of the accumulation of FDP; and (6) an acceleration of the

Table 26. Effects of glucose and/or cysteine on pathways and intermediates during incubation of S. aureus

Incubation Medium	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, µmol per h	Glucose used, µmol via HMP	Glucose used, µmol via EM	µmol FDP/g	µmol NAD/g
None	0	17.6	82.4	2.0	8.6	1.5	7.1	<1.0	6.4
PBG	2	8.0	92.0	0.9	7.6	0.6	7.0	136.3	4.8
PBG+100mM CYS-HCl	2	2.7	97.3	0.05	5.4	0.1	5.3	<1.0	3.3
Buffer only	2	15.7	84.3	2.9	8.0	1.3	6.7	<1.0	6.3
Buffer+100mM CYS-HCl	2	0.2	99.8	0.02	5.0	T	5.0	<1.0	4.8

S. aureus was grown in 2% VFCA plus 2 µg each of niacin and thiamine per ml for 17 h, in two batches. For pathways, Klett units were 266; for estimation of intermediates, Klett units were 234 and cell density during incubation was 0.07-0.08 g dry wt/100 ml incubation volume. At 0 and 2 h, pathways were estimated and phenol extracts were prepared for spectrophotometric assay of intermediates. Cysteine-HCl was dissolved in the buffer, which was then readjusted to pH 7.0. PBG, 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose. (One experiment.)

decrease in the level of NAD. To this list may be added (7) an inhibition of the increase in the level of ATP during incubation (Tables 23-D, 27-A, and 28-B), and (8) an apparent decrease in viability of the cells.

This last effect was suggested by two types of observations. At the conclusion of most of the later incubation experiments, a loopful of incubation mixture was streaked onto one-half of a TSA plate. This allowed a rough semi-quantitative estimate of the viability of the cells after the incubation period, as each incubation flask contained a similar quantity of cells within each experiment. The results of this rough approximation suggested that most of the cells, perhaps as much as 99%, were non-viable following incubation in PBG plus cysteine. This effect of cysteine was observed in at least 5 or 6 out of 10 experiments. The cell densities during incubation were quite high, so that a slight decrease in viability would not be detected by this rough method. Nevertheless, the decrease in viability that was observed was essentially complete. The second type of observation involved the use of viable counts. S. aureus was incubated in PBG with or without 30 mM cysteine-HCl. The cell density during incubation was about 0.08 g dry wt/100 ml. At hourly intervals, dilutions of the incubation media up to 10^{10}-fold were made in water and 0.1 or 1.0 ml were placed into sterile plastic petri dishes, followed by 15-16 ml sterile TSA, which had been kept as a liquid at about 40C. For cells incubated without cysteine, the zero time counts were too low (the medium had been over-diluted), but at 1, 2, and 3 h, the counts were 4.5, 4.0, and 2.0 x 10^9 cells/ml. The decrease in viability from 1 to 3 h was thus about 56%. In contrast, for cells incubated with cysteine, the counts were 4.8 x 10^9 cells/ml at zero time, but the remaining counts were zero because of overdilution of the medium.

The results suggested that at least 99% of the cells were killed by incubation in the presence of cysteine. This rough approximation from the viable counts seemed to confirm what had been suggested by the previous observations, namely, that incubation of S. aureus in PBG plus cysteine resulted in an increase in the loss of viability of these cells.

Measurement of the FDP levels in these cells showed <1.0 at zero time, 126.6 μmol/g in the cells incubated in PBG after 3 h, and only 6.0 μmol of FDP/g after incubation in PBG plus 30 mM cysteine-HCl (Table 25, part B). Thus, the presence of cysteine allowed an increase of FDP that was only 5% of the control. The levels of NAD in these cells were 6.8 μmol/g at zero time, 4.4 μmol/g after 3 h of incubation in PBG, and only 0.7 μmol/g when cysteine was present in the PBG. The decreases in NAD were 35% in the control and 90% with cysteine added.

Comparing the data, the cells that had lost about one-half of their viability accumulated over 120 μmol of FDP/g, whereas those that had lost more than 99% of their viability accumulated only 6 μmol of FDP/g. This suggested that cysteine, not the high level of FDP, was involved in the loss of viability. To summarize the effects of cysteine on intermediates during incubation of S. aureus, FDP accumulated to 132.1 ± 26.0 μmol/g during incubation of VFCA-grown cells in PBG (8 experiments), and to 8.7 ± 6.3, 3.1 ± 2.7, and <1.0 ± 0.0 during incubation in PBG plus 30, 60, and 100 mM cysteine, respectively (3, 2, and 3 experiments; $p < 0.001$). In 7 experiments in which cells were grown in VFCA and incubated in PBG, the level of NAD decreased from 7.2 ± 0.8 μmol/g at 0 time to 4.8 ± 0.6 μmol/g after 3 h of incubation ($p < 0.001$). In the presence of 10 and 30 μmol cysteine/ml, the levels of NAD were 1.3 ±

0.6 and 1.2 ± 0.8 μmol/g in 3 experiments after 3 h of incubation. The percentage decreases in NAD after incubation with 0, 10, and 30 mM cysteine were 33.7 ± 7.1, 79.8 ± 9.3, and 84.2 ± 8.8%, respectively ($p<0.001$).

During incubation in PBG, the level of ATP in cells grown in VFC and VFCA increased by 13.0 ± 5.2 μmol/g (7 experiments), and in VFCA-grown cells 13.2 ± 3.2 μmol/g (3 experiments). Addition of 10 and 100 mM cysteine allowed an increase of 4.4 ± 0.9 μmol/g (2 experiments). In cells grown in synthetic medium, the increases in ATP were 17.9 ± 2.1 and 8.1 ± 0.6 μmol/g without and with 100 mM cysteine, respectively (2 experiments). For the effect of cysteine on both groups of cells, $p<0.05$.

8. 2-Mercaptoethanol and dithiothreitol

The effects of two other reducing agents, 2-mercaptoethanol (2ME) and dithiothreitol (DTT) were compared with those of cysteine (Table 27). Incubation of cells in PBG plus 50 or 250 mM 2ME resulted in an inhibition of the HMP pathway and of the TCA cycle. The accumulation of FDP with 50 and 250 mM 2ME was both higher (137%) and lower (85%) than the control (100%), suggesting that 2ME was acting similarly to cysteine (30%). Effects on the levels of NAD and ATP in the presence of 2ME were also observed. Thus, 2ME had effects similar to those of cysteine, but higher concentrations of 2ME were required.

In contrast to 2ME, incubation of cells in PBG plus DTT over a 10-fold concentration range had relatively little effect on the pathways and only slightly reduced glucose utilization (Table 27, parts A and B). The effects of FDP varied, with increases of about 118% (part A) and 66% (part B) compared to the control (100%).

Therefore, cysteine and 2ME had generally similar effects on path-

Table 27. Effects of sulfhydryl reagents on pathways and intermediates during incubation of S. aureus

Expt.	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	% HMP	% EM	TCA ACT.	Glucose used, μmol per h	Glucose used, μmol via HMP	Glucose used, μmol via EM	Intermediates, μmol /g G6P	Intermediates, μmol /g FDP	Intermediates, μmol /g NAD	Intermediates, μmol /g ATP
(A)	None	0	19.5	80.5	2.3	7.2	1.4	5.8	<1.0	<1.0	6.2	4.1
	None	3	15.1	84.9	1.6	5.0	0.8	4.2	1.6	91.6	4.2	15.0
	10mM Cysteine-HCl	3	12.4	87.6	0.3	3.0	0.4	2.6	<1.0	27.6	1.7	9.2
	50mM 2ME	3	9.3	90.7	0.7	3.8	0.4	3.4	<1.0	125.2	3.9	8.8
	250mM 2ME	3	5.1	94.9	0.3	3.2	0.2	3.0	1.3	77.5	<1.0	4.7
	10mM DTT	3	13.5	86.5	1.5	4.6	0.6	4.0	1.3	109.3	4.2	13.6
	100mM DTT	3	16.7	83.3	1.2	4.0	0.7	3.3	<1.0	108.1	4.4	13.5
(B)	None	0	20.6	79.4	2.0	8.3	1.7	6.6	---	<1.0	7.2	---
	None	3	14.5	85.5	1.1	6.6	1.0	5.6	---	137.9	4.7	---
	100mM DTT	3	10.7	89.3	0.8	5.8	0.6	5.2	---	90.7	4.6	---

S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (part A, 260 Klett units; part B, 252 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At 0 and 3 h, pathways were estimated and phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation: part A, 0.08-0.09 g dry wt; part B, 0.05 g dry wt per 100 ml total. 2ME, 2-mercaptoethanol. DTT, dithiothreitol.

ways and intermediates in non-growing S. aureus, in contrast to the effects of DTT. The number of reducing equivalents per molecule (1 each for cysteine and 2ME, 2 for DTT) may be a factor in these differences.

9. Iodoacetate

The observed accumulations of FDP, with much smaller accumulations of DHAP, suggested that a possible site of inhibition in the EM pathway was the enzyme glyceraldehyde-3-phosphate dehydrogenase. The sensitivity of this enzyme to inhibition by the alkylating agent, iodoacetic acid (IAA), suggested that incubation of S. aureus in PBG plus IAA would be appropriate. The results of two such experiments are presented as Table 28. In the presence of iodoacetate, the accumulation of FDP was an average of 1.9 times higher than in the control without IAA. DHAP increased 1.4 times more with than without IAA, but the level of GAP was unaffected (Table 28, part A).

Table 28, part B, shows effects of both IAA and of cysteine on cells grown in Edamin and incubated in PBG. The increase of FDP in the presence of IAA was 8-fold higher than in the absence of IAA. DHAP was also increased by IAA. Unexpectedly, the level of ATP decreased in the presence of IAA, compared to a 2.6-fold increase in the control without IAA.

The cysteine used routinely in this investigation was the HCl salt, which was acidic in solution. Concentrations of 30 and especially 100 mM cysteine-HCl required neutralization, and this was accomplished by addition of KOH. Neutralization of 30 or 100 mM cysteine-HCl thus required approximately 30 or 100 mM KOH, thus resulting in an incubation medium that was also 30 or 100 mM in KCl. To avoid this problem, cysteine was

Table 28. Effects of cysteine or iodoacetate on levels of intermediates during incubation of S. aureus

Expt.	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	Intermediates, μmol/g G6P	FDP	DHAP	GAP	NAD	ATP
(A)	None	0	---	<1.0	<1.0	<1.0	9.0	---
	None	2.5	---	137.0	9.8	1.0	6.6	---
	0.05 mM IAA	2.5	---	281.8	15.0	1.0	9.4	---
	0.5 mM IAA	2.5	---	236.7	13.2	1.0	9.9	---
(B)	None	0	<1.0	<1.0	<1.0	<1.0	---	14.5
	None	2	1.4	26.6	2.8	<1.0	---	38.0
	0.1 mM IAA	2	2.8	215.1	8.7	<1.0	---	7.7
	30 mM CYS-HCl	2	<1.0	2.1	1.8	<1.0	---	9.4
	30 mM CYS-FB	2	<1.0	2.8	1.6	<1.0	---	8.0

S. aureus was grown in media supplemented with 2 μg each of niacin and thiamine per ml for 17 h: part A, 2% VFC, 326 Klett units; part B, 2% Edamin, 338 Klett units. The cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At 0 time and at the end of incubation, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation: 0.10-0.12 g dry wt/100 ml total. IAA, iodoacetate, Cysteine-HCl and cysteine-FB (free base) were dissolved in the buffer, which was then readjusted to pH 7.0 with KOH.

added as the free base to the incubation medium. The results of incubation of S. aureus in 30 mM cysteine-HCl or cysteine-free base are shown in Table 28, part B. The respective increases in FDP were 8% and 10% of the control. Although this difference was not great, the cells incubated in the presence of KCl exhibited a smaller increase of FDP. Cysteine also caused a reduction in the level of ATP in these cells.

Recalling the data of Table 14, it was found that NaCl seemed to reduce the observed levels of FDP and the reduction was directly related to the length of exposure of the cells to NaCl. The data in Table 16, part B, also show an effect of salt, comparing incubation in the presence of 100 mM cysteine-HCl (and 100 mM KCl) with 100 mM cysteine-free base. The increases of FDP were 6% and 15% of the control in the presence and in the absence of KCl, respectively. Although this difference was not significant, it was consistent with the observed effect of NaCl found previously (Table 16).

Therefore, iodoacetate exerted an effect on the level of FDP during incubation, causing a significant increase over a level that was already unusually high. Incubation in the presence of IAA did not apparently affect the levels of NAD, but did cause a reduction of ATP.

10. Carbonyl cyanide m-chlorophenyl hydrazone

A recurring observation in this investigation was the apparent relationship between the levels of FDP and ATP. The data in Table 12 indicated that incubation of S. aureus in a non-Pi buffer with increasing concentrations of Pi yielded parallel increases of both FDP and ATP. Similarly, data in Table 20 showed that incubation in non-Pi buffers without added Pi resulted in much smaller increases in both FDP and ATP.

Hence, it appeared that the increases in the levels of both FDP and ATP depended on a source of Pi.

Incubation in the presence of cysteine also affected ATP levels, as was shown in Tables 16, 23, 27, and 28. The average increase in ATP (in 5 experiments) was 3.0-fold (range 2.6-3.7). Incubation in the presence of 10, 30, and 100 mM cysteine resulted in no more than a 2.2-fold increase (section 7, cysteine).

In an attempt to more firmly establish a relationship between FDP and ATP, cells were incubated in PBG with or without the uncoupling agent, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The results (Table 29) showed major differences caused by CCCP. The level of ATP in the control without CCCP increased by a factor of 2.5. But in the presence of 0.01 and 0.1 mM CCCP, ATP remained near the zero time level. The increases of FDP in the presence of 0.01 and 0.1 mM CCCP were only 19% and 8%, respectively. Thus, CCCP clearly had parallel effects on the increases of both FDP and ATP in the non-growing cells.

11. Amino acids and the reversal of the accumulation of FDP

After observing that three amino acids could prevent the accumulation of FDP during incubation, and before it was known that cysteine itself would do the same, the next question was whether the amino acids could actively reverse the accumulation of FDP after it had occurred.

S. aureus was incubated in PBG with or without arginine, cysteine, and glutamine. After 2.25 h, one-half of the cells that had been incubated in PBG only were transferred to PBG plus the amino acids, and one-half of the cells that had been incubated in PBG plus amino acids were transferred to PBG without the amino acids. The results (Table 30)

Table 29. Effects of an uncoupling agent on levels of intermediates during incubation of S. aureus

Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	Intermediates, μmol/g G6P	FDP	DHAP	ATP
None	0	<1.0	<1.0	<1.0	8.2
None	2	2.0	195.3	5.8	20.4
0.01 mM CCCP	2	1.0	37.9	2.8	9.5
0.1 mM CCCP	2	<1.0	16.1	1.5	7.1

S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (165 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At 0 and 2 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation was 0.05-0.06 g dry wt/100 ml total. CCCP, carbonyl cyanide m-chlorophenyl hydrazone, was dissolved in 95% ethanol and then added to the incubation flasks. The same volume of ethanol was added to the control. Final concentration of ethanol was about 0.2%. (One experiment.)

Table 30. Effects of the change of incubation medium on levels of intermediates in S. aureus

	Addition to 0.05 M K-Pi buffer, pH 7.0, plus 1% glucose	Incub. Time, h	Intermediates, μmol/g G6P	FDP	DHAP	ATP
	None	0	---	<1.0	<1.0	---
(1)	None	2.25	1.0	84.8	3.1	31.2
	Arg, cys, gln	4.5	<1.0	23.0	3.2	27.2
(2)	Arg, cys, gln	2.25	<1.0	11.1	3.7	15.4
	None	4.5	<1.0	57.2	4.1	17.7

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h, washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose (PBG), either without (1) or with (2) amino acids for 2.25 h. At 2.25 h, one-half of each set of cells was harvested, washed once in buffer, and added to PBG with (1) or without (2) amino acids, and incubated for an additional 2.25 h. At 2.25 and 4.5 h of incubation, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities during incubation were 0.07-0.08 g dry wt/150 ml. Total volumes were 300 ml at 2.25 h and 150 ml at 4.5 h. Arg, L-arginine-HCl, 50 mM; cys, L-cysteine-HCl, 30 mM; gln, L-glutamine, 200 mM. (One experiment.)

indicated that the large accumulation of FDP in the cells in PBG (84.8 μmol/g) decreased by almost 75% (to 23.0 μmol/g) in the presence of the amino acids. In the opposite experiment, after FDP had increased to only 11.1 μmol/g during incubation in PBG plus the amino acids, the level of FDP than increased by more than 5-fold when the amino acids were eliminated from the incubation medium.

To more firmly establish the effects of amino acids on the reversal of the accumulation of FDP, the experiment was repeated with the addition of two controls, which were to leave a portion of each set of cells in the original medium (PBG without or with amino acids) for the entire period of incubation. The results in Figure 4 show that the level of FDP increased to about 115 μmol/g after 2 h in PBG and continued to increase (to 151 μmol/g) after an additional 2 h in PBG. The same cells transferred to PBG plus amino acids showed a decrease of FDP to about 5 μmol/g. In the reverse experiment, the cells incubated in PBG plus amino acids had only 4.3 and < 1.0 μmol FDP/g at 2 and 4 h, respectively, while in the portion of cells transferred to PBG without amino acids, FDP increased to 73 μmol/g

Therefore, these results clearly show that (i) the observed accumulation of FDP was not a permanent, irreversible occurrence, and (ii) that the amino acids could not only prevent but also actively reverse the accumulation of FDP.

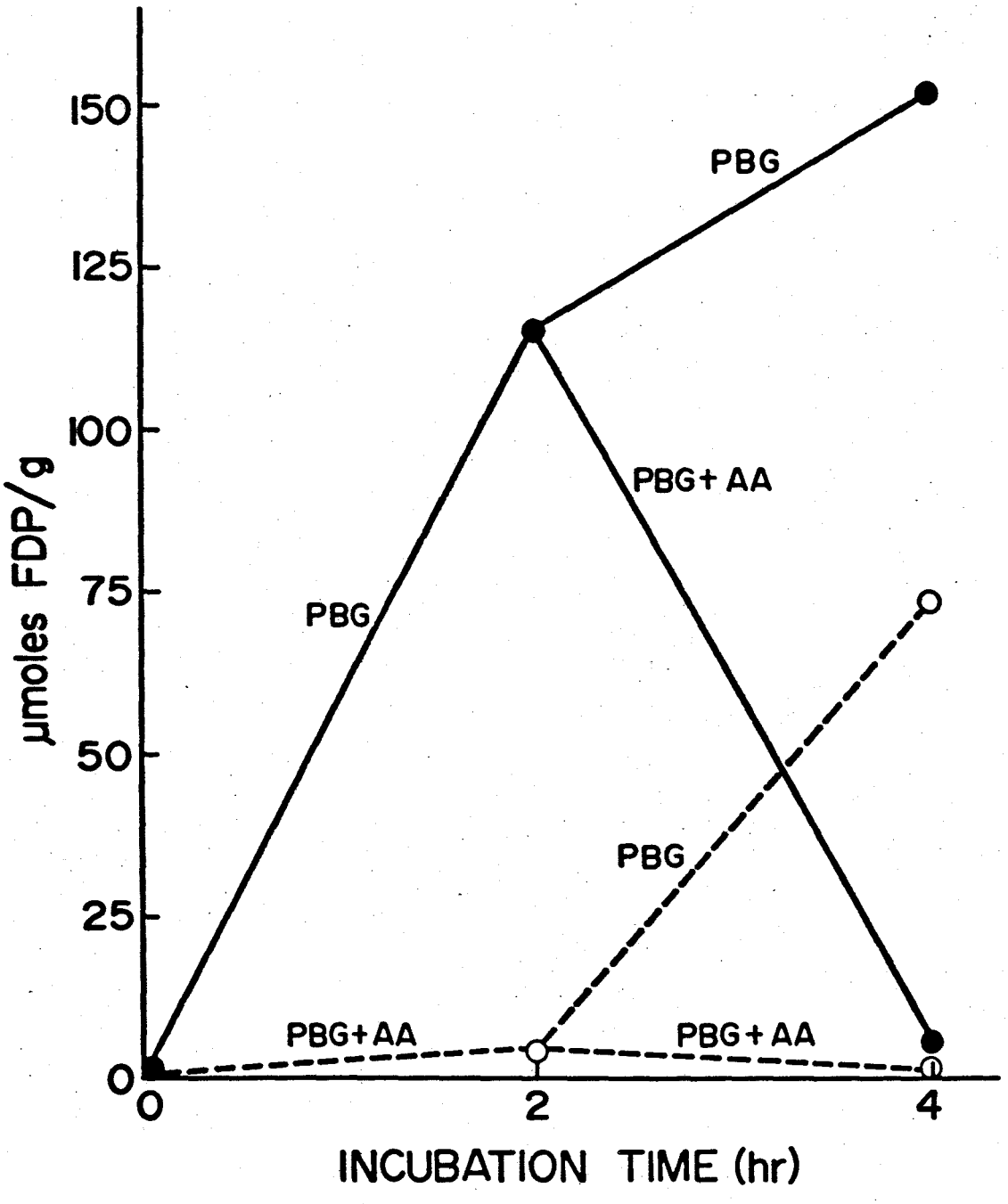
12. Lack of effect of chloramphenicol on the accumulation of FDP

As indicated previously, FDP levels in growing cells were consistently low (less than 1 μmol/g). The accumulation of FDP occurred only when cells were incubated in Pi buffer plus a carbohydrate.

Figure 4. Effect of a change of incubation medium on FDP levels in S. aureus

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (293 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose, with or without amino acids. At 2 h, one-third of the cells were removed from the media, washed once, and placed into new incubation medium, without and with amino acids, and incubated for an additional 2 h. Also at 2 h, 1/3 of the cells were removed for phenol extraction, while 1/3 of the cells remained in the original incubation media. At 4 h, phenol extracts of the four final sets of cells were prepared for spectrophotometric assay of FDP. Cell densities during incubation were 0.08-0.10 g dry wt/100 ml incubation medium. Total volumes were 300 ml for the 2 h extraction and 100 ml at 4 h. (One experiment.)

Figure 4. Effect of a change of incubation medium on FDP levels in S. aureus



Under these conditions, the cells were not able to grow (i.e., multiply) because S. aureus requires 10-15 amino acids for growth. Hence, the incubated cells were termed non-growing cells. However, lack of growth would not necessarily prevent the breakdown and resynthesis of enzymes with altered regulatory mechanisms that might result in the observed accumulation of FDP. Therefore, to preclude protein synthesis, cells were incubated in PBG with and without 100 μg of chloramphenicol/ml. Cells were also incubated in PBG plus arginine, cysteine, and glutamine with or without chloramphenicol. The results (Table 31) showed that the presence of chloramphenicol made essentially no difference in the accumulation of FDP, either in the presence or the absence of the amino acids.

13. Effects of cysteine during simulation of the pathways estimation conditions

After establishing some of the effects of cysteine on S. aureus during incubation, it was desirable to test some of these effects on cells under conditions designed to resemble those used for the estimation of the pathways. A similar experiment was designed to examine the effects of the glucose concentration during incubation, comparing the glucose concentration used for the pathways estimation (6.25 mM) with that used in the standard incubation (1% or about 56 mM). (This was presented in section III, B, 2 and in Figure 2).

To simulate the pathways estimation conditions, each addition was multiplied by 20 to yield 20 ml of 0.1 M K-Pi buffer, pH 7.0, 50 ml of cell suspension (1200 Klett units), and 10 ml of glucose solution (9 mg/ml or 0.05 M), in a total volume of 80 ml. The concentrations of glucose and Pi under pathways estimation conditions were thus 6.25 mM and

Table 31. Lack of effect of chloramphenicol on the accumulation of FDP during incubation of S. aureus

Addition to 0.05 M K-Pi buffer, pH 7.0 plus 1% glucose	Incub. Time, h	μmol FDP/g
None	0	<1.0
None	3	89.5
100 μg CA/ml	3	87.9
Arg, cys, gln	3	11.3
Arg, cys, gln, plus CA	3	12.5
2 mM $MgCl_2$	3	87.4
0.5 mM dibutyryl cAMP	3	87.8

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (171 Klett units), washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the indicated media. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell density during incubation was 0.09-0.11 g dry wt/100 ml total. CA, L-chloramphenicol, 100 μg/ml, final concentration. Arg, L-arginine-HCl, 50 mM; cys, L-cysteine-HCl, 30 mM; gln, L-glutamine, 200 mM. The Na salt of N^6, $O^{2'}$-dibutyryl-adenosine-3',5'-monophosphate (cyclic AMP) was used. (One experiment.)

56.25 mM, respectively. (The glucose and Pi concentrations during the standard incubation were about 56 mM (1%) and 50 mM, respectively.)

The results of the pathways simulation are presented as Table 32. In the control without cysteine, the FDP concentrations at 1 and 2 h were 51.6 and 45.2 μmol/g, respectively, suggesting that the maximum accumulation was probably higher than either of these values and that the concentration had reached a maximum between 1 and 2 h of incubation (compare with Figure 2). The accumulations of FDP in the estimation medium plus cysteine at 1 and 2 h were 2% and 6% of the control, respectively. The results of this experiment seemed to confirm what had been observed previously, that incubation in the presence of cysteine inhibited glucose utilization. The control cells used an average of 1.5 times more glucose than the cells incubated in the presence of cysteine.

These results suggested that the effects of incubation of S. aureus, whether under pathways estimation conditions or the standard incubation, were similar. FDP accumulated and NAD decreased under both conditions. Furthermore, the effects of cysteine were also similar under both sets of conditions, i.e., the accumulation of FDP was inhibited and the decrease of NAD was augmented.

In order to examine the effects of cysteine directly on the pathways, cysteine was added directly to the estimation medium under the standard, non-growing conditions. To compare the effects of cysteine on non-growing and growing cells, the estimation medium was supplied with growth medium (VFCA at 2% final concentration). The results of two experiments are presented as Table 33. The presence of cysteine

Table 32. Effects of cysteine-HCl on intermediates and glucose utilization during incubation of S. aureus under conditions similar to those used for the estimation of the pathways

Incubation Medium	Incub. Time, h	µmol FDP/g	µmol NAD/g	Glucose, mg used	Glucose used, %
None	0	<1.0	5.7	--	—
(1) Estimation Medium	1	51.6	4.7	30.3	30.5
Estimation Medium	2	45.2	4.2	65.8	66.2
(2) Estimation Medium +100 mM CYS-HCl	1	1.4	4.2	23.1	22.3
Estimation Medium +100 mM CYS-HCl	2	2.7	3.9	43.2	41.7

S. aureus was grown in 2% VFCA plus 2 µg each of niacin and thiamine per ml for 17 h (245 Klett units), washed once in 0.05 M K-Pi buffer, resuspended in the same buffer, and adjusted to a density of 1250 Klett units (a 1:10 dilution equalled 125 Klett units). In this incubation (scaled up 20-fold compared to the pathways estimation conditions), the estimation medium consisted of: 20 ml 0.1 M K-Pi buffer, pH 7.0; 50 ml of the 1250-Klett unit cell suspension; and 10 ml of glucose (9 mg/ml or 0.05 M; final concentration 6.25 mM), in 250 ml flasks. The flasks were incubated in a water bath at 37 C with shaking at 100 oscillations/min. Phenol extracts for spectrophotometric assay of intermediates were prepared at 0, 1, and 2 h of incubation. Cell density during incubation was 0.09-0.10 g dry wt/80 ml total. Glucose was measured by the anthrone test. (One experiment.)

Table 33. Effects of cysteine during estimation of pathways in growing and non-growing S. aureus

Estimation Conditions	% HMP	% EM	TCA ACT.	% glucose used	Glucose used, μmol per h	Glucose used, μmol via HMP	Glucose used, μmol via EM
(A)Non-growing	14.4	85.6	7.0	64.5	8.1	1.2	6.9
+30mM CYS-HCl	25.4	74.6	2.6	57.5	7.3	1.8	5.5
+100mM CYS-HCl	25.7	74.3	1.4	56.5	7.2	1.8	5.4
Growing	27.8	72.2	1.2	68.1	8.6	2.4	6.2
+30mM CYS-HCl	24.6	75.4	1.5	67.3	8.4	2.1	6.3
+100mM CYS-HCl	26.1	73.9	1.5	66.3	8.2	2.1	6.1
(B)Non-growing	19.7	80.3	2.3	66.4	8.4	1.6	6.8
+30mM CYS-HCl	25.4	74.6	0.5	52.0	6.6	1.7	4.9
+100mM CYS-HCl	26.3	73.7	0.4	47.9	6.2	1.6	4.6
Growing	27.7	72.3	0.6	62.3	7.8	2.2	5.6
+30mM CYS-HCl	36.3	63.7	1.0	45.6	5.8	2.1	3.7
+100mM CYS-HCl	38.8	61.2	0.9	44.3	5.4	2.1	3.3

S. aureus was grown in 2% VFCA plus 2 μg each niacin and thiamine per ml for 17 h (part A, 242 Klett units; part B, 245 Klett units) and washed once in 0.05 M K-Pi buffer, pH 7.0. Pathways were estimated under non-growing conditions as described in Methods, and under growing conditions provided by the addition of VFCA to the estimation medium at 2% final concentration. Cysteine-HCl was dissolved in the buffer and neutralized with KOH. The cells in part A were the same as those used for Table 31; those in part B were suspended in 0.05 M K-Pi buffer, pH 7.0, at 1250 Klett units and kept at 4 C for about 3 h prior to estimation of the pathways.

under non-growing conditions stimulated both the percentage and amount of glucose used via the HMP pathway. The EM pathway and the TCA cycle underwent a slight decrease in activity, and the percentage of total glucose used also decreased slightly.

In contrast, cysteine had essentially no effect on growing cells (Table 33, part A), but as shown in part B, the % HMP pathway increased and the amount of glucose utilized (both the total and that amount via the EM pathway) decreased. These changes may have been caused by storage (and probably starvation) of the cells in buffer at 4 C for about 3 h prior to estimation of the pathways.

Cysteine apparently had contrasting effects on non-growing cells. If cells were incubated with cysteine and the pathways were estimated without cysteine, the effects noted previously (e.g., decrease in the HMP pathway, the TCA cycle, and glucose utilization) occurred during the estimation of the pathways. In contrast, the results in Table 33 suggested that the presence of cysteine during the pathways estimation caused a stimulation of the HMP pathway, and only slight reductions in the TCA cycle and glucose utilization.

For comparison, the data of Table 16, part B may be recalled. Two contrasting situations may be considered. Cells were incubated in PBG and pathways were estimated without cysteine (line C) and cells were incubated and pathways were estimated in the presence of 100 mM cysteine (line d). The only differences between these cells were in the TCA cycle (much lower in the presence of cysteine) and in glucose utilization (the total used and the amount used by the EM pathway were slightly lower in the presence of cysteine). Incubation in cysteine

also inhibited the accumulations of both FDP and ATP. Furthermore, estimation of the pathways at zero time in the presence (line b) or the absence (line a) of cysteine had similar effects: inhibition of the TCA cycle and a slight decrease of glucose utilization in the presence of cysteine.

Therefore, the effects of cysteine on the pathways may be summarized: S. aureus incubated in PBG plus cysteine, followed by estimation of the pathways without cysteine demonstrated (i) decreased percentage of glucose oxidation via the HMP pathway, (ii) decreased total glucose utilization, (iii) decreased glucose utilization via the HMP pathway, and (iv) decreased TCA cycle activity. (Other effects included decreased accumulation of FDP and ATP, augmented decrease of NAD, and decrease in viability.) Estimation of the pathways in the presence of cysteine, whether at zero time or after incubation with cysteine, resulted in (i) slight stimulation of the HMP pathway, (ii) a slight decrease in the total glucose used, (iii) a slight decrease in glucose utilization via the EM pathway, (iv) a small increase in glucose utilization via the HMP pathway, and (v) a considerable decrease in the activity of the TCA cycle.

The only major differences appeared to be a slight stimulation of the HMP pathway in the presence of cysteine, accompanied by a slight decrease of the EM pathway. Total glucose utilization and the activity of the TCA cycle decreased whether cysteine was present or not, although the activity of the TCA cycle was much lower when cells were incubated with cysteine followed by the pathways estimation without cysteine.

14. Anaerobic conditions

The facultatively anaerobic nature of staphylococci required at least a preliminary examination of the effect of anaerobiosis on levels of FDP. The results of three experiments are presented as Table 34. Growth of S. aureus under anaerobic conditions followed by incubation in 0.1 M K-Pi buffer, pH 7.0, plus 1% glucose, resulted in an accumulation of FDP to only 14.4 μmol/g. (No comparison with aerobically-grown cells was made, but an accumulation of FDP to as much as 60-100 μmol/g would have been expected.) This experiment suggested that anaerobic growth of S. aureus tended to inhibit the accumulation of FDP. For part B, cells were grown aerobically in the media indicated. Because the anaerobic conditions required incubation of flasks without shaking, the aerobic flasks were also incubated without shaking, which probably explains the relatively low accumulation of FDP (15.6 μmol/g) that occurred in the control. The differences in the aerobic and anaerobic incubations were small, suggesting that the aerobic conditions were really semi-anaerobic and were not sufficiently aerobic to cause a significant accumulation of FDP in the control, and that the accumulations of FDP primarily reflected the growth conditions.

Because the stationary incubation of the aerobic flasks probably caused the smaller accumulation of FDP, a series of flasks was incubated under conditions of decreasing aerobiosis, from aerobic shaking to anaerobic stationary (Table 34, part C). The levels of FDP attained with shaking and stirring were similar and resembled the levels usually obtained under these conditions. The levels in the aerobic stationary and anaerobic flasks were much higher than previously, possibly because of the mixing of the incubation mixtures before and after anaerobic incubation, and the exposure to air for several minutes prior to harvesting.

Table 34. Effects of anaerobiosis on levels of FDP in S. aureus

Expt.	Growth Conditions	Growth Medium (Klett units)	Incubation Conditions	Incub. Time, h	μmol FDP/g
(A)	Anaerobic	VFC+pyruvate +uracil (43)	Aerobic	0	<1.0
				3.5	14.4
(B)	Aerobic	VFC (290)	None	0	<1.0
			Aerobic	3	15.6
			Anaerobic	3	13.4
		VFC without Thiamine (91)	None	0	<1.0
			Aerobic	3	1.3
			Anaerobic	3	<1.0
		VFC+5% NaCl (181)	None	0	<1.0
			Aerobic	3	10.8
			Anaerobic	3	18.8
		TSB (333)	None	0	<1.0
			Aerobic	3	<1.0
			Anaerobic	3	1.2
(C)	Aerobic	VFC (296)	None	0	<1.0
			Aerobic		
			-Shaking	3	100.3
			-Stirring	3	107.5
			-Stationary	3	64.6
			Anaerobic	3	74.0

S. aureus was grown in the indicated medium, aerobically for 17 h, anaerobically for 44 h. 2% VFC was supplemented with 2 μg each of niacin and thiamine per ml except where indicated. Pyruvate (Na salt), 20 mM final concentration; uracil, 1 mM final concentration. TSB, Trypticase Soy broth, 3% solution, unsupplemented. Anaerobic growth and anaerobic incubation conditions were as described in Methods. All cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in 0.1 M K-Pi buffer, pH 7.0, plus 1% glucose. At 0 time and at the end of incubation, phenol extracts were prepared for spectrophotometric assay of FDP. Cell densities during incubation: part A, 0.03 g dry wt; part B, 0.02-0.07 g; part C, 0.09-0.10 g/100 ml.

Note on incubation conditions: part A, shaking at 200 rev/min; part B, aerobic, flasks were stationary at 37 C; anaerobic, flasks were stationary in an anaerobic incubator at 37 C; part C, aerobic stirring was provided by slow rotation of a 1 1/2" Teflon-coated stirring bar.

Nevertheless, these anaerobic levels of FDP were about 33% lower than the aerobic levels.

These results suggested that anaerobic growth and anaerobic incubation of S. aureus tended to inhibit the accumulation of FDP during incubation.

15. Comparison of several Staphylococcus strains

S. aureus Towler had been used exclusively in this investigation and in order to determine the occurrence of the accumulation of FDP among staphylococci, five other strains, both coagulase positive and coagulase negative, were tested. The results in Table 35 indicated that the accumulation of FDP in non-growing cells was not unique to the Towler strain. Even among the six strains of staphylococci, there was a five-fold range in the accumulation of FDP, from a low of 46 to a high of 232 μmol/g, the highest level of FDP observed. Two other features of this experiment require mention. One was the suggested relation between growth of the organisms and the extent of accumulation of FDP. The two strains with the poorest growth, Peoria and UA 724, exhibited the greatest accumulations of FDP, and conversely, the strains with the best growth, B VIII and B McK, had the smallest increases in the level of FDP. (Compare with results in section III, A, 7, Growth medium.) The second feature involved the coagulase-positive B VIII, and B McK, a coagulase-negative mutant of B VIII. The similar accumulations of FDP in these strains suggested that coagulase production was not directly related to glucose catabolism, at least to the extent of affecting FDP production. The 1.4 μmol of FDP/g in the Towler strain was the only instance of a zero time level of FDP greater than 1 μmol/g.

Table 35. Levels of FDP in six staphylococcal strains during incubation

Strain	Growth (Klett Units)	Incub. Time, h	μmol FDP/g	μmol NAD/g
S. aureus				
Towler	276	0	1.4	3.0
		3	95.8	2.9
Peoria	229	0	< 1.0	2.4
		3	215.8	1.6
BVIII	299	0	< 1.0	3.2
		3	46.3	2.6
S. epidermidis				
7292	264	0	1.6	1.3
		3	99.3	< 1.0
UA 724	192	0	1.2	7.2
		3	232.5	3.6
B McK	307	0	< 1.0	2.8
		3	49.7	2.7

The 6 strains were grown in 2% VFC plus 2 μg each of niacin and thiamine for 17 h, washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in the same buffer plus 1% glucose. At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Cell densities during incubation were 0.06-0.10 g dry wt/100 ml incubation medium. (One experiment.)

These results suggested that the accumulation of FDP and, hence, the cause for such an accumulation, were probably common to staphylococci in general.

16. Comparison with other bacteria

The apparent ubiquity of the accumulation of FDP among staphylococci required that a comparison be made with other bacterial species. Those chosen were Bacillus cereus T and clinical strains of Escherichia coli and Pseudomonas aeruginosa. The results of incubation of these organisms in Pi-buffered glucose or glycerol are presented as Table 36. Other than S. aureus, only B. cereus exhibited any significant accumulation of FDP, but it was only about 6% of that in S. aureus.

These results suggested that after growth of organisms in VFC and incubation in phosphate-buffered glucose or glycerol, only S. aureus and, to a much smaller extent, B. cereus, exhibited an accumulation of FDP. This indicated the existence of major metabolic differences between these four organisms, at least with regard to the production and/or utilization of FDP. Therefore, in the system used here, the staphylococci may be unique among microorganisms with regard to the actual cause of the accumulation of such high levels of FDP. The next section of Results will present data which suggests that a probable cause of the accumulation of FDP may be an inhibition of the activity of glyceraldehyde-3-phosphate dehydrogenase resulting from the incubation of S. aureus in Pi-buffered glucose.

Table 36. Levels of intermediates in four bacterial species during incubation

Organism	Growth (Klett Units) Starter	Main	Carbohydrate	Incub. Time, h	Intermediates, μmol/g FDP	PEP	Pyruvate	NAD
Staphylococcus aureus	56	298	None	0	<1.0	1.6	<1.0	6.0
			Glucose	3	58.9	1.0	1.8	---
			Glycerol	3	15.9	<1.0	1.3	4.4
Bacillus cereus	163	400	None	0	<1.0	<1.0	<1.0	1.8
			Glucose	3	3.6	<1.0	<1.0	1.6
			Glycerol	3	<1.0	1.4	<1.0	1.6
Escherichia coli	215	282	None	0	<1.0	<1.0	<1.0	1.6
			Glucose	3	<1.0	1.2	<1.0	<1.0
			Glycerol	3	<1.0	2.5	1.4	1.6
Pseudomonas aeruginosa	168	415	None	0	<1.0	<1.0	1.8	1.3
			Glucose	3	<1.0	<1.0	1.9	---
			Glycerol	3	<1.0	<1.0	2.1	---

The organisms were transferred from TSA plates to starter flasks of 2% VFC for 7 h of growth, then 5 ml were transferred to the main flasks for 17 h of growth. S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine; the other organisms were grown in unsupplemented 2% VFC. The cells were washed once in 0.05 M K-Pi buffer, pH 7.0, and incubated in 0.1 M K-Pi buffer, pH 7.0, plus 1% glucose (w/v) or 1% glycerol (v/v). At 0 and 3 h, phenol extracts were prepared for spectrophotometric assay of intermediates. Extracts of E. coli (3 h) and Pseudomonas (0 and 3 h) were treated with DNAse plus 10-25 μmol of $MgCl_2$ at 37 C and centrifuged at 13,000 rev/min for 15 min. Cell densities during incubation were: S. aureus, 0.07 g dry wt; others, 0.10-0.15 g dry wt/100 ml total. (One experiment.)

IV In vitro studies of enzymes

The previous section dealt with in vivo observations concerning a number of factors that affected the accumulation of FDP during incubation of non-growing S. aureus in phosphate-buffered glucose. Such results provided primarily circumstantial evidence regarding the actual cause of the increase of FDP. To more firmly establish a reason for such an accumulation of a metabolic intermediate, the relationship between enzyme activity and intermediate levels had to be examined, based on the supposition that the inhibition of one or more enzymes during incubation resulted in the observed accumulation of FDP. This section will present results of assays of several enzymes in crude or partially purified extracts of S. aureus. The results obtained from assays of glyceraldehyde-3-phosphate dehydrogenase (GAPD) have direct implications with regard to the accumulation of FDP.

A. FDP aldolase

The large accumulation of FDP suggested initially that FDP aldolase may have been involved, directly or indirectly. In an attempt to determine a possible relationship between the FDP accumulation and the activity of FDP aldolase, several features of this enzyme were observed.

(1) Assay of a crude extract of cells grown in TSB demonstrated the importance of arsenate in the assay system. The control, using the standard assay, yielded 85.8 mU/mg (0.06 mg protein). Elimination of arsenate yielded 20.2 mU/mg, replacing 20 μmol arsenate by 50 μmol Pi allowed only 6.7 mU/mg, and the presence of both arsenate and Pi resulted in 74.2 mU/mg. Pi apparently was inhibitory to the assay, possibly by inhibiting the GAPD reaction.

(2) An observation that had implications with regard to the gel assay of FDP aldolase was that ammonium persulfate, used to polymerize the gels, inhibited the spectrophotometric assay of aldolase by completely inhibiting the GAPD reaction. A standard assay of FDP aldolase from a crude extract of cells grown in TSB yielded 46.3 mU/mg (0.26 mg protein) and 46.4 mU/mg (0.53 mg protein). The presence of 5 μmol of ammonium persulfate initially and the addition of 5 μmol of ammonium persulfate during the reaction completely inhibited the observed activity of FDP aldolase. Assay of the commercial preparation of GAPD yielded 297.8 mU/mg (0.08 μmol GAP) and 361.0 mU/mg (0.16 μmol GAP). However, 5 μmol of ammonium persulfate, both present initially and added during the reaction, completely inhibited the enzyme. Therefore, running gel 2 (photopolymerized) replaced running gel 1 (polymerized using ammonium persulfate) for electrophoresis of both FDP aldolase and GAPD.

(3) Some preliminary observations were made on the effect of EDTA on FDP aldolase. In these experiments, a sample of extract was incubated in the presence of 0.01 or 0.1 M EDTA in an ice bath for up to 1 h. Assay of these extracts (Table 37) suggested that FDP aldolase was sensitive to EDTA. The two auxiliary enzymes, TPI and GAPD, were probably not affected by EDTA because the commercial enzymes were prepared in 0.5 and 1.0 mM EDTA, respectively.

Further evidence for the effect of EDTA on FDP aldolase was obtained by coupling aldolase to the oxidation of NADH. The presence of 50 μmol EDTA initially allowed an activity which decreased from 36.2 to 18.6 mU/mg, compared to 91.8 mU/mg in the control without EDTA. (The commercial α-glycerophosphate dehydrogenase used in this assay was prepared in about 0.27 mM EDTA.)

Table 37. Effect of EDTA on activity of FDP aldolase in extracts of S. aureus

Assay No.	Cell Growth Medium	Extract	mg Protein Assayed	Units TPI/Assay	μmol EDTA/Assay	Specific Activity -EDTA	Specific Activity +EDTA	% Inhibition
1	VFC	Crude	0.5, 1.0	100	0.8	24.5	16.9	31.0
					8.0		16.5	32.6
2	TSB	Crude	0.1	50	0.4	93.3	57.7	38.2
					4.0		64.0	31.4
3	TSB	Crude	0.1, 0.25	20	0.2	75.3	62.2	17.4
					2.0		60.5	19.6
					4.0		56.0	25.6
					0.4		57.5	23.6
4	TSB	Supernate (Dialyzed)	0.02, 0.04	20	0.4	269.4	215.0	20.2
					4.0		231.0	14.2

S. aureus was grown in either 2% VFC plus 2 μg each of niacin and thiamine per ml (294 Klett units) or in unsupplemented 3% TSB (361 Klett units) for 17 h, washed once in 0.1 M Tris buffer, pH 7.5, and treated for preparation of cell-free extract as described in Methods. FDP aldolase was assayed by the standard procedure described in Methods, except for the units of triosephosphate isomerase (TPI) indicated. The extract was incubated in the presence of EDTA (0.01 or 0.1 M) for up to 1 h before assaying for aldolase. The μmol of EDTA indicated were the final amounts in the 1 ml of assay volume.

These results suggested that some of the FDP aldolase activity in S. aureus was provided by a form of the enzyme which was inhibited by EDTA.

(4) The results of FDP aldolase assays of an ammonium sulfate fractionation of a crude extract of S. aureus are shown as Table 38. Although the total yield was less than satisfactory, the majority of the activity (about 21%) was apparently in the supernatant fluid.

(5) Ammonium sulfate (A.S.) also had an effect on the observed activity of FDP aldolase. Prior to dialysis to remove A.S., the 70-100% A.S. fraction (Table 38) had an activity of 27.7 mU/mg (range 26.0-31.5 mU/mg, using 0.03, 0.06, and 0.12 mg protein and 200 U TPI per assay). After dialysis, the same fraction yielded 40.2 mU/mg (Table 38), an increase of 45%, or, using 200 U TPI, 32.0 mU/mg (0.04 mg protein), an increase of 16%.

Dialysis of the supernatant fraction had a greater effect on the activity of FDP aldolase. Prior to dialysis, activity was not proportional to protein concentration, ranging from 199.0 mU/mg (0.04 mg protein) to 47.7 mU/mg (0.3 mg protein). After dialysis, the specific activity increased considerably, to an average of 324 mU/mg (Table 38) and became more proportional to the amount of protein assayed. For example, one series of assays yielded activities ranging from 402 mU/mg (0.02 mg protein) to 294 mU/mg (0.08 mg protein), and a second series yielded 364 mU/mg (0.02 mg protein) and 343 mU/mg (0.06 mg protein).

(6) The effects of arginine, cysteine, and glutamine on the accumulation of FDP required that the effects of these amino acids on FDP aldolase be tested. The results of a series of assays are shown as Table 39. Arginine and glutamine over a 0-100 μmol/ml range had essentially no effect (series 1, 3, and 4). Increasing concentrations of

Table 38. Ammonium sulfate fractionation of S. aureus and assays of FDP aldolase

Fraction	Volume ml	Protein mg/ml	Total Protein mg	Total Activity U	Sp. ACT. mU/mg	Purification Factor	Yield %
Crude extract	31.3	12.6	394.4	34,392	87.2(5) (76.1-111.7)	1.0	100.0
0-40% A.S.	4.0	2.1	8.4	903	107.5(1)	1.2	2.6
40-70% A.S.	3.0	10.9	32.7	742	22.7(5) (19.8-31.0)	0.3	2.2
70-100% A.S. (Dialyzed)	10.5	1.9	20.0	804	40.2(9) (35.5-46.4)	0.4	2.3
Supernate (Dialyzed)	24.3	0.9	21.9	7,091	323.8(15) (241.0-526.0)	3.7	20.6

S. aureus was grown in TSB (Difco, 30 g/l) for 17 h (361 Klett units), washed once in 0.1 M Tris buffer, pH 7.5, sonicated, and fractionated with enzyme grade ammonium sulfate (A.S.) as described in Methods. For dialysis, 5.5 ml of the 70-100% A.S. fraction and 10.0 ml of supernate were dialyzed twice against 500 ml each of 0.05 M Tris buffer, pH 7.5, for 3 h each, yielding the final volumes indicated. FDP aldolase was assayed by the procedure described in Methods. Figures in parentheses indicate the number of assays and the range of specific activity.

Table 39. Effects of cysteine, arginine, and glutamine on the activity of FDP aldolase from S. aureus

Assay series	μmol Cysteine-HCl	μmol Arginine-HCl	μmol Glutamine	Specific Activity mU/mg	% of Activity
1	0	0	0	90.1	100
	0	10	0	79.8	89
	10	0	0	73.4	82
	0	0	10	72.3	80
2	0	0	0	93.9*	100
	10	0	0	106.3*	113
	50	0	0	69.5*	74
	100	0	0	46.5*	49
3	0	0	0	80.0	100
	0	10	0	89.4	112
	0	50	0	88.6	111
	0	100	0	84.4	106
4	0	0	0	96.0	100
	0	0	10	92.0	96
	0	0	50	96.6	101
	0	0	100	89.2	93
5	0	0	0	72.4	100
	10	10	10	69.6	96
	50	50	50	37.6	52
	100	100	100	40.4	56

S. aureus was grown in 2% VFC plus 2 μg each of niacin and thiamine per ml for 17 h (328 Klett units), washed once in 0.05 M Tris buffer, pH 7.5, suspended in 0.05 M K-Pi buffer, pH 7, and incubated in 0.05 M K-Pi buffer, pH 7, + 1% glucose for 3 h. Cells were then washed once in 0.05 M Tris buffer, pH 7.5, and suspended in 0.1 M Tris buffer, pH 7.5, for preparation of cell extract by sonication. Crude extract (4.0 ml) was dialyzed against 500 ml of 0.05 M Tris-HCl buffer, pH 7.5, for 24 h. FDP aldolase was assayed by following NADH oxidation, as described in Methods. For assays 1 and 2, 0.3 mg protein was used; assays 3-5, 0.05 mg protein. Amino acid solutions were neutralized to pH 6-7 before use. *Mean of two assays.

cysteine, however, caused a reduction in activity of up to 51% at 100 μmol cysteine/ml (series 2). Series 5 showed a similar reduction in activity which was probably caused by cysteine.

(7) The activity of FDP aldolase in spectrophotometric assays was generally proportional over as much as a 6-fold range of protein concentration. For example, a dialyzed crude extract of cells grown in VFC yielded 88.7 mU/mg (0.03 mg protein), 100.8 mU/mg (0.05 mg), 86.2 mU/mg (0.1 mg), and 90.8 mU/mg (0.2 mg); average 91.6 mU/mg. Assays of crude extract and A.S. fractions of cells grown in TSB were also proportional over as much as a 4-fold range of protein concentration.

(8) Assay of FDP aldolase in polyacrylamide gels revealed 2 or 3 bands of activity. Extracts from S. aureus Towler and Peoria and from S. epidermidis 7292 yielded one band that migrated with the marker dye (Rf=0.96-1.0), suggesting a relatively low molecular weight, possibly corresponding to the activity observed in the supernatant fluid from the A.S. fractionation (Table 38). A gel stained for protein indicated the presence of a substantial amount of protein at this position. These preliminary experiments also revealed the presence of another band at Rf 0.8-0.9.

Because of the probable lack of a direct relationship between FDP aldolase and the accumulation of FDP, this enzyme was not examined further.

B. Glyceraldehyde-3-phosphate dehydrogenase

Several factors, including the effects of iodoacetate, which greatly augmented the accumulation of FDP during incubation, suggested that GAPD be assayed in extracts of S. aureus.

(1) Initial assays were done in an attempt to optimize the system. As mentioned previously, because of the nature of the preparation of the substrate GAP, 1 μmol was used as the standard amount in the assay. This amount may not have been optimum, as indicated by the lack of proportionality between activity and protein concentration. Increasing amounts of NAD did not significantly affect the activity. Using 0.8 mg of protein from a crude extract of cells grown in VFC, the specific activities were 2.6, 3.1, 3.4, and 3.2 mU/mg with 1, 4, 7, and 10 μmol NAD, respectively. Therefore, 1 μmol of NAD was used routinely. The effects of 10-40 mM arsenate and 2-20 mM cysteine indicated that maximum activity was obtained with 10 μmol of arsenate and 5 μmol of cysteine.

The requirement for cysteine was indicated by the initial assays of GAPD. Assays of a dialyzed crude extract of cells grown in VFC yielded no activity until a solution containing the three amino acids (about 15 μmol arg, 9 μmol cys, and 60 μmol gln) was added, and the activity increased from 0 to 65.6 mU/mg. Subsequent assays revealed that only cysteine was essential for activity of GAPD.

(2) Preliminary assays of GAPD indicated that Pi inhibited activity. A number of assays of GAPD (10 or 20 mM arsenate and 5 or 10 mM cysteine) indicated that addition of 25 or 50 μmol of Pi reduced the activity by 37-88%. Under standard conditions of 10 mM arsenate and 5 mM cysteine, the inhibition by Pi was 60-88%. Replacing 10 μmol of arsenate by 50 μmol of Pi resulted in a reduction of activity from 187.7 mU/mg to 32.0 mU/mg (after 8 min), compared to 175.3 mU/mg in the control, which remained essentially constant over the same period.

These results indicated that Pi could replace arsenate in the

GAPD assay because Pi is the natural substrate. However, in contrast to arsenate, Pi may have allowed accumulation of product (1,3-diphosphoglycerate), which then caused deceleration of the reaction.

(3) The effects of the amino acids on the accumulation of FDP and the relationship between FDP and GAPD necessitated that the effects of the amino acids on GAPD be examined. The results of a series of assays of GAPD in the presence of cysteine, arginine, and glutamine are shown as Table 40. Series 1 indicated that 10 μmol each of arginine and glutamine had no effect. Series 2 indicated that neither arginine nor glutamine could replace cysteine, and that cysteine was essential for activity. Although arginine was inhibitory at 16 μmol (series 3), neither arginine nor glutamine had a significant effect at 100 μmol (series 6).

The requirement for cysteine for activity of GAPD was a consistent observation. Several assays of extracts from VFC-grown cells yielded minimal or no activity in the absence of cysteine followed by increases of up to 17 mU/mg after addition of 10 μmol of cysteine. Therefore, cysteine was essential for activity of GAPD.

(4) As shown previously, the presence of 2-mercaptoethanol (2ME) during incubation of S. aureus in PBG had a slightly inhibitory effect on the accumulation of FDP (Table 27). The results of assays of GAPD (Table 41) indicated that 2ME could replace cysteine; but to be as effective, the concentration of 2ME had to be 4-8 times higher than that of cysteine. In contrast to the lack of effect of dithiothreitol (DTT) on the accumulation of FDP (Table 27), DTT was more effective than cysteine in the GAPD assay (Table 41). Considerable activity of GAPD was observed at a concentration of DTT as low as 0.05 μmol/ml.

Table 40. Effects of cysteine, arginine, and glutamine on activity of GAPD from S. aureus

Assay Series	µmol CYS	µmol ARG	µmol GLN	Initial Specific Activity mU/mg	% of Initial Activity	Additions µmol	Final Specific Activity mU/mg
1	5	0	0	12.5	100	50 Pi	2.7
	5	10	0	12.5	100	50 Pi	3.4
	5	0	10	12.5	100	50 Pi	2.5
	5	10	10	12.5	100	50 Pi	2.8
2	10	0	0	18.8	100	50 Pi	2.9
	0	10	0	0.2	1	0.1 CYS	1.9
	0	0	10	0.2	1	1.0 CYS	13.9
3	0.2	2	0	9.9	76*	5 CYS	17.4
	0.2	16	0	3.0	23	5 CYS	17.9
	0.2	0	2	9.7	75	5 CYS	17.4
	0.2	0	16	9.2	71	5 CYS	15.6
4	0.2	0	0	3.2	100	0.2 CYS	7.9
	0.2	2	0	3.3	103	0.2 CYS	7.8
	0.2	8	0	2.4	75	0.2 CYS	6.8
	0.2	16	0	2.1	66	0.2 CYS	6.3
5	0.2	0	0	14.1	100	None	---
	0.2	10	0	8.8	62		
	0.2	0	10	9.6	68		
	0.2	10	10	5.6	40		
6	0.2	0	0	12.0	100	5 CYS	37.6
	0.2	100	0	9.6	80	5 CYS	28.8
	0.2	0	100	14.8	123	5 CYS	50.4
	0.2	100	100	15.3	128	5 CYS	38.5

S. aureus Towler was grown in 2% VFC plus 2 µg niacin and 2 µg thiamine per ml for 17 h, washed once in 0.05 M Tris-HCl buffer, pH 7.5, and suspended in 0.1 M Tris-HCl buffer, 7.5, for preparation of cell extract by sonication. Crude extract (4.0 ml) was dialyzed against 500 ml 0.05 M Tris buffer, pH 7.5, for 24 h. GAPD activity was determined by the standard assay system. For assays 1-4, 0.5 mg of protein was used per assay; assays 5 and 6, 0.1 mg of protein. Amino acid solutions were neutralized to pH 6-7 before use. CYS, cysteine-HCl; ARG, arginine-HCl; GLN, glutamine. Additions, µmol of ortho-phosphate (Pi) or cysteine, and resulting specific activity. *(Compared to 13.0 mU/mg, the average of 14.1 (series 5) and 12.0 (series 6).

Table 41. Effects of replacement of cysteine by 2-mercaptoethanol or dithiothreitol in assays of GAPD

Assay Series	mg Protein	First Addition µmol	First Addition mU/mg	Second Addition µmol	Second Addition mU/mg
1	0.5	5 CYS	13.3	None	
		10 2ME	6.8		
2	0.1	5 CYS	46.2	None	
		20 2ME	36.2		
		5 DTT	43.8		
		25 DTT	48.6		
3	0.2	None	5.2	20 2ME	77.8
		5 CYS	93.2		
4	0.03	None	0.0	5 CYS	177.0
		10 CYS	160.7	6 H_2O	92.3
		2 2ME	34.7	10 2ME	106.0
		10 2ME	124.7	10 2ME	123.7
5	0.1	5 CYS	98.1	6 H_2O	74.2
		6 2ME	40.6	20 2ME	74.2
		20 2ME	82.4	6 H_2O	63.7
		40 2ME	89.2	6 H_2O	74.2
6	0.1	5 CYS	89.4	None	
		0.05 DTT	19.3		
		0.15 DTT	32.5		
		0.25 DTT	48.2		
7	0.1	5 CYS	98.5	None	
		0.25 DTT	47.8		
		1.0 DTT	82.8		
		2.5 DTT	94.8		
8	0.1	5 CYS	98.6	None	
		5 DTT	102.9		
		25 DTT	103.4		
		50 DTT	118.4		

GAPD was assayed using the standard procedure described in Methods with the exception of the sulfhydryl reagent indicated: CYS, cysteine-HCl, 2ME, 2-mercaptoethanol; DTT, dithiothreitol. The extract used in assays 1 and 2 was from cells grown in VFC; assays 3-8, from cells grown in TSB. Series 1, dialyzed crude extract prepared before incubation of cells for 3 h in PBG (see Table 40). Series 2, dialyzed crude extract prepared after incubation of cells in PBG. Series 3, crude extract of cells grown in TSB (326 Klett units). Series 4, 0-40% ammonium sulfate fraction of the cells grown in TSB. Series 5-8, crude extract of cells grown in TSB (361 Klett units).

These results indicated that cysteine could be replaced by two other sulfhydryl reagents, 2ME and DTT, in the assay of GAPD.

(5) The effects of iodoacetate (IAA) on the accumulation of FDP (Table 28) suggested that GAPD in S. aureus may have been inhibited by this reagent. The results of six series of assays of crude extract of cells grown in VFC or TSB, and of ammonium sulfate fractions of cells grown in TSB, showed that GAPD was completely inhibited by as little as 0.5 μmol of IAA, which decreased the specific activity of 0.03 mg of protein from 0-40% A.S. fraction from 175.3 mU/mg to zero. The addition of 10 μmol of cysteine to assays containing 0.4 mg of protein from a dialyzed crude extract of VFC-grown cells could not overcome the inhibition caused by the initial presence of 1, 2, 5, or 10 μmol of IAA.

These results indicated that GAPD from S. aureus was completely inhibited by IAA in vitro, which correlated with the results of the in vivo incubation experiments.

(6) Attempts at obtaining proportionality between activity and protein concentration were generally unsuccessful. The best results were obtained with a dialyzed crude extract of VFC-grown cells: 22.6 mU/mg (0.05 mg protein), 25.0 mU/mg (0.1 mg protein), and 21.9 mU/mg (0.2 mg protein); average 23.2 mU/mg. Increasing protein concentrations of extracts of TSB-grown cells resulted in decreases in specific activity of about 50%; for example, 247.0 mU/mg (0.02 mg protein), 173.8 mU/mg (0.04 mg), 136.6 mU/mg (0.08 mg), and 112.5 mU/mg (0.12 mg protein). A possible cause for this lack of proportionality was the concentration of the substrate, GAP, at only 1 μmol/ml. Assay of a crude extract of TSB-grown cells yielded 81.2 mU/mg with 1.0 μmol GAP/assay, 230.7 mU/mg

with 2.4 μmol GAP, and 303.8 mU/mg with 4.3 μmol GAP (0.1 mg protein/assay).

(7) Assay of GAPD in polyacrylamide gels revealed two bands, a major (dark) band at Rf 0.5 and a minor (lighter) band at Rf 0.64, in extracts of cells grown in both VFC and TSB. Addition of arsenate enhanced development slightly, and 1 μmol of IAA in the presence of 0.1 μmol of cys almost completely eliminated the bands.

C. Glucose-6-phosphate dehydrogenase

The activity of glucose-6-phosphate dehydrogenase (G6PD) in extracts of S. aureus was lower than that of FDP aldolase and GAPD, generally 15-25 mU/mg in extracts of both VFCA- and TSB-grown cells. Such low activity may partially explain the greater proportionality obtained: 18.8, 21.6, 25.8, and 31.5 mU/mg with 0.12, 0.25, 0.5, and 0.74 mg protein, respectively. Assay of a crude extract of VFCA-grown cells suggested that cysteine stimulated the activity of G6PD. Assay of 0.4 mg of protein without cysteine yielded 23.3 mU/mg (average of 24.8, 19.6, and 25.4 mU/mg). Assay in the presence of 2, 8, and 16 μmol of cysteine yielded 38.4 mU/mg (average of 37.4 and 39.4), 43.0 mU/mg (average of 42.9 and 43.2), and 43.6 mU/mg, respectively.

FDP had no significant effect on the activity of G6PD from cells grown in VFCA, where the activity was 5.0 mU/mg with or without 10 μmol of FDP, using 0.9 mg of protein from a crude extract. Preliminary experiments suggested that ATP stimulated G6PD. Assay of 0.9 mg of protein from a crude extract of cells grown in VFCA resulted in 6.0 mU/mg without ATP, and 7.6, 8.5, and 9.0 mU/mg with 1, 5, and 10 μmol of ATP, respectively. The addition of 5 μmol of FDP to these assays resulted in

decreases of activity by an average of 76% (range 73-78%) at all 4 ATP concentrations, in contrast to the apparent lack of effect of FDP present initially. Essentially no activity was found when NAD replaced NADP in the G6PD assay.

D. 6-phosphogluconate dehydrogenase

The activity of 6-phosphogluconate dehydrogenase (6PGD) was generally 40-50 mU/mg in extracts of cells grown in VFCA and 150-200 mU/mg in extracts of TSB-grown cells. For example, specific activities of 146.6, 138.0, and 136.3 mU/mg were found using 0.05, 0.10, and 0.15 mg of protein, respectively, from a crude extract of cells grown in TSB. Assay of a crude extract of VFCA-grown cells yielded 55.9, 45.1, 40.8, and 34.2 mU/mg using 0.1, 0.2, 0.4, and 0.7 mg of protein, respectively.

The results of a series of assays examining the effects of FDP and cysteine on 6PGD are presented as Table 42. FDP, whether added during the reaction (series 1) or present initially (series 2), caused a significant reduction of activity. IAA was used to inhibit GAPD during the 6PGD assay, thus preventing some of the breakdown of FDP caused by FDP aldolase. Adding IAA and increasing the FDP concentration caused a further reduction of 6PGD activity (series 3). The presence of cysteine initially caused a slight stimulation of activity (series 4), but FDP, added during the reaction (series 4) or present initially (series 5 and 6) caused a significant reduction of activity. However, cysteine was able to reduce the inhibition of 6PGD by FDP (series 4) and partially reverse the inhibition by FDP (series 5 and 6). A preliminary assay (series 5) suggested that ATP reduced the inhibition caused by FDP. Essentially no activity was observed when NAD replaced

Table 42. Effects of FDP, cysteine, and ATP on activity of 6-phosphogluconate dehydrogenase from S. aureus

		First Additions			Second Additions		
Assay	mg Protein	μmol of Reagent	mU/mg	% of Activity	μmol of Reagent	mU/mg	% of Activity
1	0.2	None	44.2		5 FDP	2.5	6
2	0.3	None	42.9	(100)	2 IAA	22.9	53
		5 FDP	9.4	(22)	2 IAA	7.2	77
3	0.3	None	44.4	(100)	None	---	--
		1 FDP	16.1	(36)			
		1 FDP, 2IAA	12.4	(28)			
		5 FDP, 2IAA	7.9	(18)			
4	0.44	None	39.9	(100)	5 FDP	3.4	8
		5 CYS	40.8	(102)	5 FDP	19.2	47
		50 CYS	43.4	(109)	5 FDP	19.0	44
		100 CYS	42.2	(106)	5 FDP	18.5	44
5	0.44	None	39.0	(100)	50 CYS	19.5	50
		5 FDP	6.2	(16)	50 CYS	32.2	519
		10 ATP	34.5	(88)	50 CYS	7.4	21
		5 FDP, 10 ATP	28.0	(72)	50 CYS	7.7	28
6	0.44	None	38.5/ 26.7	(100)	6 H_2O	19.4	50
		50 CYS	41.6/ 25.9	(108)	5 FDP	16.8	40
		5 FDP	7.0	(18)	50 CYS	33.2	474
		50 CYS, 5 FDP	42.6/ 25.4	(111)	6 H_2O	16.9	40

S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (260 Klett units), washed once in 0.1 M Tris buffer, pH 7.5, and sonicated for preparation of cell extract as described in Methods. Results of assay of the crude extract are presented here. First additions indicate reagents present at the start of the reaction. The activity in assay series 6 under first additions was the initial activity (in front of the slash mark) and the final activity caused by a spontaneous change after about 4 min (after the slash mark). 6PGD was assayed by the standard procedure presented in Methods.

NADP in the 6PGD assay.

These results indicated that 6PGD from S. aureus was considerably more active than G6PD, and that it was inhibited by FDP. The in vitro effect of FDP is in contrast to the results of the in vivo incubations.

E. Pyruvate kinase

The changes in the levels of PEP during incubation suggested that pyruvate kinase (PK) be examined. PK was unique because its specific activity increased with increasing protein concentration. Assay of a crude extract of TSB-grown cells yielded 17.6, 39.5, 64.4, and 75.5 mU/mg with 0.05, 0.10, 0.15, and 0.20 mg of protein, respectively. In contrast, assay of a crude extract of cells grown in VFC resulted in 8.0, 6.8, 8.0, and 10.3 mU/mg with 0.04, 0.08, 0.16, and 0.25 mg of protein, respectively.

Preliminary assays suggested that FDP inhibited PK. Assay of 0.1 mg of protein from a crude extract of TSB-grown cells yielded 21.6 mU/mg without FDP and 23.8 mU/mg in the presence of 2 μmol of IAA and 4 μmol of Na arsenate. The presence of 10 and 16 μmol of FDP yielded specific activities of 10.1 and 8.4 mU/mg, respectively. Assay of 0.25 mg of protein from a crude extract of cells grown in VFC yielded 13.1 mU/mg with or without IAA and arsenate, and 10.1 mU/mg with 10 μmol of FDP. Addition of 10 more μmol of FDP resulted in a decrease from 10.1 to 6.0 mU/mg. Further studies of this enzyme were not done.

F. Fructose-1,6-diphosphatase

Cursory assays of fructose-1,6-diphosphatase (FDPase) in S. aureus extracts indicated that it was present and that its activity in cells grown in TSB was 2- to 5-fold higher than in cells grown in VFC. Other

observations included: (1) an initially slow reaction rate that increased to a maximum after several min; (2) Mn^{++} could replace Mg^{++} and was effective at concentrations of 0.5-3.0 μmol/ml; (3) comparable FDPase activity at pH 7.5 and 9.5; (4) 10 μmol of EDTA inhibited FDPase at Mg^{++} and Mn^{++} concentrations of 20 and 3 μmol/ml and less, respectively; (5) no apparent effect of cysteine.

V Studies on the uptake of 2-deoxyglucose during incubation of S. aureus

In order to determine the possible effects of sulfhydryl reagents on the uptake of glucose, S. aureus was incubated in 0.05 M K-Pi buffer, pH 7.0, with 2-deoxyglucose-^{14}C-UL (uniformly labeled) plus unlabeled 2-deoxyglucose (2DG) to yield an initial concentration of 1%. Cysteine was added to final concentrations of 10 and 100 mM and dithiothreitol (DTT) to a final concentration of 100 mM. To determine possible effects of an uncoupling agent on uptake, CCCP was added to a final concentration of 100 μM. The results of two experiments are presented as Figures 5 and 6. The results in Figure 5 indicated that cysteine at 10 or 100 mM had similar effects on the uptake of 2DG, and DTT had the greatest inhibitory effect on the uptake of 2DG. Figure 6 shows effects of cysteine and DTT similar to those in Figure 5. To compare the results on a percentage basis, the average of the last two points, when uptake had become essentially constant, was taken and expressed as a percentage of the control (100%). The results from Figure 5 were: control, 3198 counts/min/mg, 100%; 10 mM cysteine, 2176 counts/min/mg, 68.0%; 100 mM cysteine, 2348 counts/min/mg, 73.4%; and 100 mM DTT, 680 counts/min/mg, 21.3%. The results from Figure 6 were: control, 5319 counts/min/mg, 100%; 100 mM cysteine, 3664 counts/min/mg, 68.9%; 100 mM DTT, 948 counts/min/mg, 17.8%; and 100 μM CCCP, 1009 counts/min/mg, 19.0%.

Therefore, the uptake of 2DG in the presence of cysteine averaged 70.1% in the two experiments, and for DTT, 19.6%.

Although these values may not be directly projected to the uptake of glucose itself, the results suggested that cysteine only reduced the uptake of 2DG by 30%. The pathways simulation experiment (Table 32) showed that after 2 h of incubation, 66.2% of the glucose was used in

Figure 5. S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (182 Klett units) and washed once in 0.05 M K-Pi buffer, pH 7.0. After resuspension in the same buffer, 5 ml aliquots were added to incubation media consisting of 45 ml 0.05 M K-Pi buffer, pH 7.0 and 1% 2-deoxyglucose (●), plus 10 mM L-cysteine, free base (X), 100 mM L-cysteine, free base (■), or 100 mM dithiothreitol (Δ). At the indicated intervals, aliquots of incubation mixture were removed, filtered through a Millipore filtration apparatus using filters with 0.22 μm pore size, and washed once with 5 ml 0.05 M K-Pi buffer, pH 7.0. The filters and washed cells were placed into glass counting vials, to which were added 10 ml scintillation fluid. The activity of the cells was determined in a liquid scintillation spectrometer, and is expressed as counts/min per mg dry wt of cells. Cell density during incubation was 1.5 mg dry wt/ml, or about 0.15 g dry wt/100 ml. The amount of cells assayed was 7.4, 5.9, or 3.0 mg dry wt/sample. 2-deoxyglucose-^{14}C-UL was added to an initial activity of about 1.52 x 10^5 counts/min per ml of incubation medium.

Fig. 5. Effects of cysteine and dithiothreitol on uptake of 2-deoxyglucose- C-UL during incubation of S. aureus.

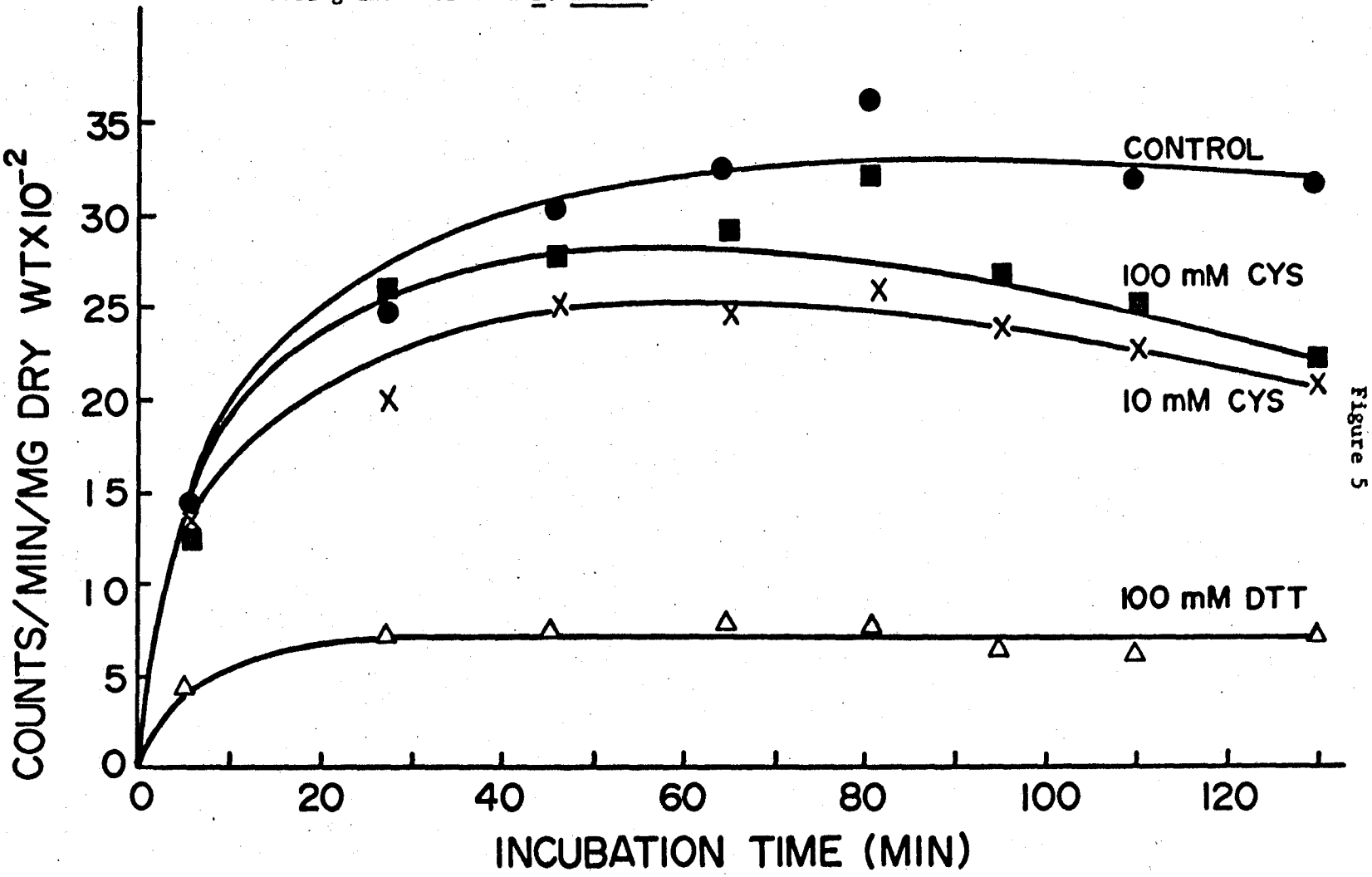


Figure 5

Figure 6. S. aureus was grown in 2% VFCA plus 2 μg each of niacin and thiamine per ml for 17 h (176 Klett units) and washed once in 0.05 M K-Pi buffer, pH 7.0. The cells were resuspended in the same buffer and 5 ml aliquots were added to incubation media consisting of 45 ml 0.05 M K-Pi buffer, pH 7.0 and 1% 2-deoxyglucose (●), plus 100 mM L-cysteine, free base (■), 100 mM dithiothreitol (Δ), or 100 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP; X). At the indicated intervals, aliquots of incubation mixture were removed, filtered through a Millipore filtration apparatus using filters with 0.45 μm pore size, and washed once with 4 ml 0.05 M K-Pi buffer, pH 7.0. The filters and washed cells were placed into glass counting vials, to which were added 10 ml scintillation fluid. The activity of the cells was determined in a liquid scintillation spectrometer, and is expressed as counts/min per mg dry wt of cells. Cell density during incubation was about 0.77 mg dry wt/ml or about 0.08 g dry wt/100 ml. The amount of cells assayed was 1.5 mg dry wt/sample. 2-deoxyglucose-^{14}C-UL was added to an initial activity of about 1.2×10^5 counts/min per ml of incubation medium.

Fig. 6. Effects of cysteine, dithiothreitol, and CCCP on uptake of 2-deoxyglucose-^{14}C-UL during incubation of S. aureus.

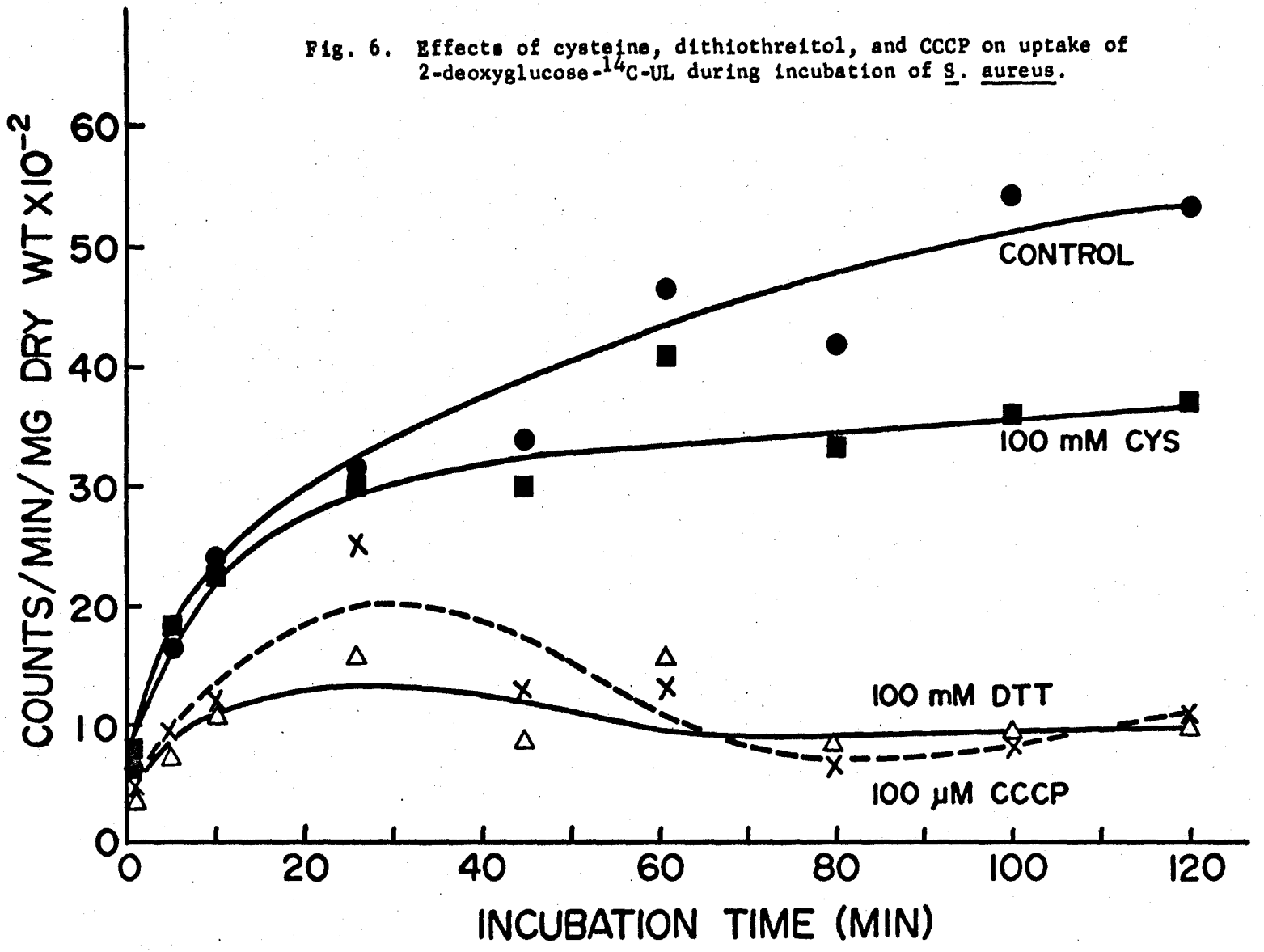


Figure 6

the absence of cysteine, and 41.7% in the presence of 100 mM cysteine. Thus, in the presence of cysteine, the cells used 63% (41.7/66.2 x 100) of the glucose that was used in the absence of cysteine. This compares favorably with the results of the 2DG uptake experiments. However, the presence of 30 or 100 mM cysteine during the actual pathways estimation (Table 33) indicated that cysteine caused much smaller reductions of glucose utilization.

The 80% inhibition of 2DG uptake in the presence of DTT may be contrasted with the effects of DTT on FDP accumulation. The results in Table 27 suggested that DTT had little effect on the pathways and caused only a slight reduction (20% and less) of glucose utilization. The accumulations of FDP in the presence of DTT also suggested little effect on glucose utilization.

The 81% inhibition of 2DG uptake in the presence of 100 μM CCCP compared favorably with the effects of CCCP on the accumulations of FDP and ATP. The results in Table 29 indicated a significant inhibition (greater than 90%) of the accumulation of FDP in the presence of 100 μM CCCP. The same concentration of CCCP also caused a reduction in the level of ATP in these cells, compared to the 2.5-fold increase in the level of ATP in the control without CCCP. These results suggested that the accumulation of FDP may be related to that of ATP in non-growing S. aureus.

DISCUSSION

This investigation has concerned two aspects of carbohydrate metabolism in Staphylococcus aureus: (i) the accumulation of FDP in non-growing cells and (ii) the effects of FDP on the HMP pathway. These problems were approached using both in vivo and in vitro methods with the intention of correlating the information obtained into a coherent description of the two phenomena.

The three major findings of this investigation are: (i) FDP accumulated to very high levels in non-growing cells incubating in Pi-buffered glucose; (ii) the control point for the FDP accumulation appeared to be GAPD; (iii) high in vivo levels of FDP apparently did not significantly affect the extent of glucose catabolism via the HMP pathway nor the activity of 6PGD. Each of these will be discussed in turn.

The first major phase of the investigation was an examination of the accumulation of FDP in non-growing S. aureus with the purposes of determining some of the factors that affected the accumulation and the basic cause for the accumulation.

During incubation of cells, FDP accumulated to very high levels during incubation in PBG, with a large variation in the levels observed: 87.3 ± 17.3 and 116.1 ± 38.6 μmol FDP/g after the cells had been grown in VFC and in VFCA, respectively (Table 4). This variability in the observed levels of FDP in the PBG controls was attributed to slight variations in the batches of growth media, small variations in the cell density during incubation, and to the inherent variability that would be expected from such large accumulations of an intermediate in vivo and the subsequent manipulations involved in preparation of the phenol

cell extracts.

The factors that affected the accumulation of FDP were divided into those present during growth of the organisms which then influenced the accumulation during subsequent incubation, and those present during the incubation only. Several factors were found to occur in both categories.

The concentration of niacin and thiamine during growth of the cells affected the accumulation of FDP during subsequent incubation (Tables 10 and 11). The standard growth medium contained 2 μg of each vitamin per ml. Lowering the concentration of vitamins during growth had opposite effects. Niacin, reduced to 0.1 μg/ml, allowed a higher accumulation of FDP, but elimination of thiamine from the medium resulted in a much lower accumulation. These effects could possibly be explained in terms of the metabolic roles of the vitamins. A lower concentration of niacin during growth resulted in lower activity of the HMP pathway and of the TCA cycle, and also resulted in less glucose catabolism via the HMP pathway and more via the EM pathway. The primary cause for this difference may have been the levels of NAD and NADP in the cells. Growth with 0.1 μg of niacin/ml resulted in initial levels of NAD and NADP that were only about 13% and 50%, respectively, as high as the levels in the cells grown with 2 μg of niacin/ml. This suggested that the intracellular NAD level was more sensitive than NADP to the concentration of niacin, which in turn suggests that the HMP pathway responded more to the concentration of NAD than to that of NADP. This confirms previous findings from this laboratory (17, 126, 127, 306). Although the level of FDP did not apparently affect the activity of the HMP pathway, these results suggested that the activity of the HMP pathway may have

influenced the level of FDP, possibly via the increased activity of the EM pathway. The greater amount of glucose catabolized by the EM pathway meant that more glucose was available to form FDP.

The effects of growth of the cells without thiamine were dramatic and surprising. Because an exogenous supply of thiamine is required for efficient operation of the TCA cycle in S. aureus (17), growth of cells in the absence of thiamine should significantly reduce the activity of the TCA cycle. Such reduced TCA cycle activity was suggested by the 2-fold higher level of PEP and the 7-fold higher level of pyruvate at zero time in the cells grown without thiamine, compared to those grown with both vitamins (Table 11). Even though these cells were grown under conditions of gluconeogenesis, there was apparently considerable formation of pyruvate and of PEP from the amino acids in the growth medium. As was shown in Table 11, in two separate experiments, the accumulation of FDP during incubation was much lower after growth of the cells without thiamine. The effect of growth in TSB on the accumulation of FDP was even greater, the level remaining below 1.0 μmol/g at all glucose concentrations.

Although growth of the cells in VFC without niacin and in TSB had a similar effect on FDP, the actual reasons for the lack of accumulation are probably different, because there are a number of differences between the two media. Data provided in the BBL Manual of Products and Laboratory Procedures (5th Edition, 1968) indicated that TSB contained niacin at a final concentration of about 0.2 μg/ml, and thiamine at a minimum final concentration of 0.01 μg/ml, which were sufficient for growth of the organisms, but considerably less than the 2.0 μg of vitamins added per ml of VFC and VFCA. Also, TSB contains 0.25% glucose.

Nolan and Nolan (213) performed an elemental analysis of Difco VFCA, and from their data, the concentrations of various elements in a 2% solution of VFCA could be calculated: phosphate (PO_4), about 2.0 mM; NaCl, about 0.1 mM; K, about 0.5 mM; Mg, about 0.4 mM. This may be compared with data provided in the BBL Manual on TSB: Pi, about 18.4 mM; NaCl, about 89.4 mM; K, about 4.1 mM; Mg, about 0.4 mM. Thus, major differences occurred between these two media in the levels of Pi and NaCl. This suggested that thiamine was not the only factor involved in the lowered accumulation of FDP during incubation.

The absence of thiamine during growth of S. aureus in VFC plus niacin was shown to result in lowered activity of the TCA cycle in a number of staphylococcal strains (17). That a decrease in the activity of the TCA cycle in S. aureus would be the major effect of a lack of thiamine suggested that the TCA cycle was involved in FDP metabolism. This problem is complicated because the TCA cycle, being an amphibolic pathway, is involved directly and indirectly in a large number of catabolic and anabolic reactions. Unfortunately, these have not been investigated in staphylococci in detail, so it is not possible to state how FDP and the TCA cycle may be related in this organism. However, it would appear that the S. aureus aerobic PFK may not be inhibited by ATP, in contrast to the mammalian enzyme (14, 186). As shown in several tables (4, 5, 12, 14, 15), the level of ATP increased by as much as 2-fold during incubation while the level of FDP also increased. It has also been observed in mammals (217) that ATP inhibited the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPD). If this were the case in S. aureus, the much higher ATP level during incubation may have contributed to the accumulation of FDP by inhibition of GAPD. Although the suggested rela-

tionship between FDP levels and the TCA cycle in S. aureus was interesting, it was not pursued further in this investigation.

Orthophosphate (Pi) is one of the most important of the inorganic constituents of cells, and is also a vital part of the energy flow between catabolic and anabolic processes in the form of the so-called "high-energy phosphates," the nucleoside di- and tri-phosphates. Therefore, it seemed proper to find that Pi had an effect on the accumulation of FDP, which was exerted during both the growth and incubation of the cells, as shown in Tables 12 and 13.

Part A of Table 12 shows that the major effect of growth in media supplemented with Pi was a lower accumulation of FDP during subsequent incubation in 0.05 M Pi buffer. There were no significant effects on the other intermediates assayed. Essentially similar results are shown in part B of Table 12, with the added results of effects on G6P and ATP. The important point of part B is that growth in the presence of added Pi affected G6P, FDP, and ATP in a similar pattern, i.e., the accumulation of these intermediates was inhibited by growth in a higher Pi concentration. This immediately suggested the existence of a Pi pool in S. aureus and prompted consideration of the levels of such a pool in cells grown in media with low and high Pi concentrations. The problem that remained, however, was how such a pool would relate to the accumulation of FDP. These possible relationships will be considered later in light of the results on the effect of Pi concentration during incubation in relation to ATP levels.

The effects of growth of S. aureus in the presence of both glucose and Pi on the subsequent accumulation of FDP were examined in one experiment. The results (Table 13) showed that growth in the presence

of glucose caused a large inhibition of the subsequent accumulation of FDP, which was partially overcome by the presence of both glucose and Pi during growth. Growth in the presence of glucose also resulted in decreased TCA cycle activity. Here again was the relationship observed previously between FDP and the TCA cycle, where lower activity of the TCA cycle occurred simultaneously with a lower accumulation of FDP. The problem still remaining is which came first, the lowered TCA cycle activity or the lower accumulation of FDP. A number of investigators (17, 42, 136, 264, 283) have demonstrated that growth in the presence of glucose causes repression of the synthesis of several TCA cycle enzymes, i.e., the "glucose effect." The actual mechanism can only be surmised until more is known about the interrelationships between the EM pathway and the TCA cycle in S. aureus.

The data in Table 14 showed some effects of NaCl on the levels of FDP. Two points are important: (i) The effect of lowering the accumulation of FDP was directly proportional to the length of exposure of the cells to salt. (ii) The effects of salt were much greater after growth in the presence of salt than after incubation in the presence of salt. If NaCl affected the permeability of the wall and/or membrane, a larger effect during growth of the cells was reasonable because the composition of these structures changes constantly during growth as both wall and membrane are synthesized. There were no effects of NaCl on the other intermediates assayed.

Arsenate has several inhibitory effects on metabolism, such as the uncoupling of substrate-linked phosphorylation of GAPD (208, 269), inhibition of respiration (47), and inhibition of the oxidation of

alpha-ketoglutarate (252). The major effect of arsenite is the inhibition of the oxidation of alpha-keto acids (140), via its effect on dihydrolipoyl dehydrogenase. The accumulation of pyruvate during incubation in the presence of these inhibitors was thus not surprising (Table 14). The two inhibitors had major effects on the accumulation of FDP during incubation. The effect of arsenate on the accumulation of FDP may be explained by the uncoupling of substrate-level phosphorylation at the GAPD step. In this process, arsenate replaces the normal substrate, Pi, resulting in the formation of 1-arseno-3-phosphoglycerate, which breaks down spontaneously to form arsenate and 3-phosphoglycerate. Thus, the carbon flow of the EM pathway remains uninterrupted, although the high-energy Pi of 1,3-diphosphoglycerate and the resulting formation of ATP are lost. Note that in the presence of both arsenate and arsenite, the accumulation of ATP was greater than without these inhibitors. This result, plus the much lower accumulation of FDP, was the earliest suggestion of the involvement of GAPD in the accumulation of FDP.

Arsenite caused an even greater accumulation of pyruvate and a lower accumulation of FDP, and thus appeared to be a more effective inhibitor of pyruvate oxidation than arsenate. The much larger accumulation of pyruvate during incubation in the presence of arsenite suggested that the TCA cycle would be inhibited. This may explain the much lower accumulation of FDP in the presence of arsenite. Thus, there have been four results suggesting that the TCA cycle was involved in FDP metabolism. Growth without thiamine, growth in the presence of glucose, and incubation in the presence of arsenate or arsenite all caused or probably caused decreased activity of the TCA cycle and a concomitant reduction in the accumulation of FDP during incubation. Of course, all four

factors may simultaneously affect processes other than those observed, which in turn may directly or indirectly affect the TCA cycle and/or the accumulation of FDP. Nevertheless, the effects were apparent and of sufficient magnitude that they were caused by direct action of the four factors rather than by experimental variability.

Another factor that was tested both during growth and incubation of S. aureus was the amino acid cysteine, which was essential for a number of staphylococcal strains (73, 166). Assay of VFCA indicated that this medium contained 0.029 μmol of cysteic acid, hence of cysteine, per ml (212). This level was apparently not limiting because Fildes and Richardson (76) found that cysteine was effective at a concentration of 10^{-6} M, and, as shown in Table 15, in two out of three experiments, there was no significant difference in growth caused by the addition of cysteine to the medium or by the use of a medium with a much higher cystine content. Also in Table 15 were shown opposite effects of the amino acid in two media. Addition of cysteine to VFCA resulted in a slightly greater accumulation of FDP during incubation, but growth of cells in Edamin, with a much higher cystine content than N-Z-Amine, resulted in a smaller accumulation of FDP. Apparently, the inherent differences in the two media had more effect on FDP accumulation than did the relatively small difference in cysteine concentration.

The growth medium itself was also found to affect the subsequent accumulation of FDP. Growth in TSB resulted in no accumulation (Table 11) and growth of S. aureus in Edamin and N-Z-Amine resulted in accumulations (Table 15) that were lower than that generally obtained after growth of cells in VFC. Differences were also observed in FDP accumu-

lation after growth of cells in VFC and VFCA (Table 12), and even between different batches of the same medium (Results, section III, A, 7). Even after growth of S. aureus in a synthetic medium, FDP accumulated during incubation, and changes in the pathways and other intermediates were similar to those in cells grown in VFC or VFCA (Table 16). The use of a defined medium eliminated problems caused by slight variations between batches of complex media and, of course, would allow for modifications of the medium to determine the effect on FDP accumulation. The effects of various growth media on the accumulation of FDP are summarized in Table 17.

The standard procedure in these experiments was to grow a batch of cells and resuspend them at a density 2-4 times higher than that attained after growth. The question arose regarding the effect of cell density on the intermediates during incubation and the effect of placing the cells in fresh growth medium on the levels of intermediates. As shown in Table 18, placing cells into fresh growth medium had an insignificant effect on the level of FDP, and the presence of glucose enhanced this increase only slightly. After 17 h of growth, the cells reached late log-early stationary phase at which time their rate of growth and metabolic activity had declined. Placing the cells into a fresh medium provided a new source of nutrients which most likely resulted in a renewal of growth and perhaps the temporary establishment of higher steady-state levels of intermediates, including FDP. An increase in the level of FDP in fresh growth medium suggested that the levels of intermediates may also have undergone change during the 17 h growth of the organisms, i.e., the FDP level may have been higher at

6 h and then declined by 17 h. Opheim and Bernlohr (219) found that during the growth of Bacillus licheniformis in a medium containing 15 mM glucose, the decrease in the intracellular concentration of G6P was concomitant with the disappearance of glucose from the medium. It was reasonable for the level of G6P in the cells to decrease when its source was exhausted. However, S. aureus was usually grown in the absence of glucose, with amino acids serving as the carbon source, necessitating extensive use of the EM pathway in the gluconeogenic direction. In a study of a related problem, Szynkiewicz et al. (285) examined pentose biosynthesis in E. coli growing on glucose or acetate and found that cells grown on acetate synthesized most of their pentoses via transketolase and transaldolase, using the non-oxidative portion of the HMP pathway. During growth of E. coli on glucose, the oxidative decarboxylation of 6PG predominated. An analysis of this problem by Katz and Rognstad (148) indicated that the net flow of carbon was from hexose to pentose in the oxidative branch and from pentose to hexose in the non-oxidative branch. This suggests that cells growing gluconeogenically would not be expected to have very large steady-state pools of G6P or 6PG. Therefore, during the 17 h growth of S. aureus, the steady-state levels of the EM pathway intermediates may vary slightly. However, 12-h cells behaved just like 17-h cells in the accumulation of FDP (K. Edwards, unpublished results).

A larger number of factors were found to affect FDP accumulation during incubation.

Although technically not a factor during incubation nor a metabolic factor, the washing of the cells after incubation had a significant effect on the observed levels of FDP. As shown in Table 7, three washes

reduced the intracellular levels of FDP by about 50% and of 6PG by as much as 86%. The procedure adopted thereafter eliminated this variable. An effect of harvesting was observed by Wimpenny and Firth (320), who found that the total NAD and NADH in Klebsiella aerogenes decreased by as much as 90% during harvesting of the cells from a tryptone growth medium. They indicated that a factor in such a decrease may have been the rapid destruction of NAD and NADH in non-growing cells. Iizuka and Mizuno (135) observed a turnover of NAD, but not of NADP, in growing S. aureus, and in the present investigation, the results of R. Hoo (126) were confirmed, namely that NAD breakdown was observed in non-growing cells. A study by Roberts and Wolffe (244) indicated that E. coli was able to absorb labeled FDP from the medium, and the appearance of label in the nucleic acid fraction suggested that such absorbed FDP entered the pathways of glucose metabolism. Studies by Winkler (323) and by Dietz (56) have shown that hexose phosphates can be directly transported into S. aureus and other organisms.

Of the factors that affected the accumulation of FDP during incubation, perhaps the most direct was the ultimate source of FDP, the glucose in the incubation medium. The results of an experiment on the effects of two glucose concentrations are presented as Fig. 2. This experiment demonstrated that the extent of accumulation of intermediates was directly related to the exogenous glucose concentration and that the accumulation of intermediates reversed spontaneously, apparently as a result of the diminished exogenous glucose concentration. This reversal of the accumulation of the intermediates to zero-time levels agreed with the complete lack of accumulation of intermediates in cells

incubated without glucose. The results of a related experiment are shown in Table 11, part A. These results again indicated that the extent of FDP accumulation was directly related to the exogenous glucose concentration. This suggests that at least two factors affected the accumulation of FDP: (i) the rate of uptake of glucose and (ii) the rate of the formation and utilization of FDP. Considering these factors in more detail, there are probably at least five processes operating: (i) the rate of uptake of glucose from the medium; (ii) the relative rates of glucose utilization by the EM and HMP pathways; the rates of (iii) formation and (iv) utilization of FDP; and (v) the ability of the cells to contain high levels of FDP and other intermediates.

It is reasonable to suppose that higher exogenous glucose concentrations would allow more rapid uptake of glucose by the cells until the capacity of the transport mechanism became saturated. Saturation of a glucose transport mechanism of S. aureus was observed by Egan and Morse (71), who found that the uptake system became saturated at 5 mM glucose and lactose, and at about 50 mM maltose. A similar study was done on E. coli by Kaback (141), who used α-methylglucoside. Thus it is apparent that carbohydrate transport mechanisms have a saturation level, which would tend to limit the rate of utilization of glucose and, hence, the formation of any intermediates of glucose catabolism.

It may be appropriate here to consider the possible relationship between glucose utilization and the levels of PEP in non-growing S. aureus. During incubation of cells in PBG, the intracellular level of PEP decreased (Tables 9, 10, 11, 18, 36 and Fig. 2). These cells had been grown in an amino acid medium without added carbohydrate, so the

PEP-HPr system for carbohydrate transport was not significantly utilized. Subsequent incubation of these cells in PBG resulted in the rapid uptake of glucose, which required utilization of PEP. Hence, the level of PEP decreased during incubation, in contrast to the levels of the other EM pathway intermediates assayed.

Concerning the relative rates of glucose utilization by the EM and HMP pathways, the results consistently indicated that both the percentage and the amount of glucose catabolized by the HMP pathway decreased during incubation of cells. This was accompanied by a considerable reduction in the level of NAD, whereas NADP remained constant or increased slightly. This was partially explained by the recent finding in S. aureus of isozymes of G6PD and 6PGD that were active with both NAD and NADP (200). If the level of NAD decreased, the activity of the limiting enzyme, whether G6PD or 6PGD, would decrease, thus lowering the activity of the HMP pathway.

The role of the HMP pathway in metabolism must also be considered in relation to the decreasing activity of the HMP pathway during incubation of S. aureus. The HMP pathway is involved primarily in biosynthesis, both when cells are growing glycolytically or gluconeogenically. In cells growing in the presence of glucose, the oxidative portion of the HMP pathway (measured in the pathways estimation) provides the biosynthetic reducing agent, NADPH, and 5-carbon intermediates. The non-oxidative portion provides various 3-,4-, and 5-carbon carbohydrate precursors. Under gluconeogenic conditions, the non-oxidative reactions function similarly, while the oxidative portion may operate at a reduced rate because of the absence of exogenously-supplied carbohydrates and the resulting relatively low levels of G6P and 6PG. In non-growing

cells, the requirement for biosynthetic precursors would be greatly reduced or non-existent. The presence of G6PD and 6PGD means that intermediates entered the non-oxidative portion of the HMP pathway, but because the intermediates could not be used for synthesis, they had at least two possible fates: to accumulate or to be converted to intermediates of the EM pathway, namely, fructose-6-phosphate (F6P) and/or GAP. With the greater activity of the EM pathway relative to that of the HMP pathway, the GAP produced by the HMP pathway would probably be metabolized to PEP, which would then be further metabolized to pyruvate directly or via the PEP-HPr system for glucose transport. The F6P produced by the HMP pathway may have contributed to the accumulation of FDP. Whatever may have been the actual case, it remains that the activity of the HMP pathway decreased under non-growing conditions. Model and Rittenberg (198) observed a decrease in activity of the HMP pathway in E. coli entering the stationary phase of growth, or when the nitrogen source was exhausted, or during methionine deprivation. The latter conditions were not too different from those in this investigation, where S. aureus was placed into PBG without a source of nitrogen.

Two other processes that determined the levels of accumulation of FDP were the rates of formation and utilization of FDP in the non-growing cells. The rate of formation of FDP would have been determined by the activities of the enzymes in the EM pathway leading to FDP, which included the PEP-HPr system for the simultaneous transport and phosphorylation of glucose to form G6P. Although the cells were grown under conditions of gluconeogenesis, the enzymes of the EM pathway are constitutive and the extent of accumulation of FDP certainly indicated that the

enzymes in the early reactions of the EM pathway were active.

A factor that must be considered is the presence of FDPase in these cells. An active FDPase would tend to reduce the accumulation of FDP during incubation. However, several factors may indicate a lack of such an effect in S. aureus. (i) The cells were incubated in the presence of a high concentration of glucose after having been grown under conditions of gluconeogenesis. Therefore, the existing control mechanisms were established for such growth, and when the cells were placed into the incubation medium, which allowed only minimal protein synthesis, if any, new mechanisms appropriate for glycolysis could not be established. Such a sudden change, from growth to non-growth and from gluconeogenesis to glycolysis, apparently was a significant factor in the accumulation of FDP. (ii) The high concentration of FDP during incubation may have inhibited the FDPase in S. aureus, as it did in Candida utilis (248). (iii) Although AMP was considered to be a major inhibitor of FDPase (84, 231), the enzymes from some organisms have been shown to be inhibited by ATP (205, 272). The high and increasing levels of ATP in S. aureus during incubation may possibly have contributed to an inhibition of FDPase.

The rate of utilization of FDP during incubation would be determined primarily by the activities of the enzymes distal to FDP in the glycolytic EM pathway. These consist of FDP aldolase and the five enzymes from GAPD to pyruvate kinase, the latter group known as the constant proportion group of enzymes (227). As will be discussed subsequently, it was probably the inhibition of one of these enzymes during incubation that resulted in the accumulation of FDP.

Finally, an item that must not be neglected was the ability of the cells to contain such high concentrations of FDP. Levels of 87.3 $\pm$

17.3 and 116.1 ± 38.6 μmol of FDP/g (after growth in VFC and VFCA, respectively) were observed, and accumulations to 200 μmol of FDP/g (44 mM) and more were observed in several experiments. Although an attempt was made to measure the level of FDP in the supernatant fluid after several washings of the cells, during which the intracellular FDP level decreased, very little FDP was detected, possibly because of phosphatases which may have converted FDP to F6P during its passage out of the cells. Nevertheless, the very high intracellular levels of FDP suggested that little if any leakage occurred during the incubation period. The amounts of FDP observed may thus be taken as minimal levels. It is possible that bacterial membranes and/or walls are relatively impermeable to diphosphorylated sugars compared to monophosphorylated sugars. Moses and Sharp (204) assayed intra- and extracellular FDP and triose phosphate in three strains of E. coli and found that in exponentially-growing cells, the amount of extracellular FDP was never more than 35% of the total, and was usually much less. In contrast, the amount of extracellular triose phosphate ranged from 34% to 100%. After nitrogen starvation, the amount of extracellular FDP was zero, with one exception, while the extracellular triose phosphate ranged from zero to 100%. Thus, E. coli is relatively resistant to leakage of FDP, but not of triose phosphate. The 200 μmol of FDP/g (44 mM) observed in S. aureus could possibly have been exceeded by a longer incubation time and/or higher glucose concentration. All of the processes discussed here, and possibly others, acted in concert to control the extent of accumulation of FDP during incubation of S. aureus in PBG.

As shown in Table 19 and in Fig. 3, a number of carbohydrates other

than glucose were able to cause an accumulation of FDP. The effects of these carbohydrates on FDP were not unexpected because staphylococci are capable of metabolizing a variety of sugars (5, 6, 39, 236). Although the utilization of mannitol is one of the characteristics of S. aureus (5), the relatively low accumulation of FDP during incubation in the presence of mannitol may be explained by the observations of Murphey and Rosenblum (206), who found that the enzymes for mannitol utilization were inducible. Galactose was very effective in causing an accumulation of FDP. Bissett and Anderson (11) found a pathway for galactose degradation consisting of isomerization of galactose-6-P to tagatose-6-P, phosphorylation with ATP, and cleavage of the resulting tagatose-1,6-diphosphate to DHAP and GAP by a special aldolase (not FDP aldolase).

Of the several effectors of FDP accumulation, glucose and Pi were the most direct, as indicated by data in Tables 8, 9, 11, and 12. The data of Tables 12 and 20 indicate that Pi had major effects not only on FDP, but also on ATP during incubation of the non-growing cells. This suggested relationship between FDP and ATP may not be surprising because ATP provides one of the two phosphates in FDP via PFK. The data in Table 12, part B and Table 20, part A show similar patterns of FDP accumulation with increasing concentrations of Pi during incubation. Table 12, part B also shows some effects of Pi on ATP. In the cells grown without Pi and incubated in Tris-maleate buffer plus glucose, the level of FDP remained low and the level of ATP decreased during incubation. In the cells grown with Pi and incubated in Tris-maleate buffer plus glucose, the level of FDP increased (to over 14 μmol/g) and that of ATP approximately doubled. Note also in Table 12, part B and in Table 20, part B, that the levels of FDP and ATP parallel one another

in all incubation conditions, in Pi and non-Pi buffers. These results suggested that Pi was required for the accumulations of both FDP and ATP during incubation. Other experiments involving sulfhydryl reagents and an uncoupling agent also strongly suggested a relationship between the levels of ATP and FDP in the non-growing cells. This will be considered in detail later during the discussion of the mechanism of accumulation of FDP.

Deliberation on the cause of the FDP accumulation led to a comparison of the growth and incubation media. FDP did not accumulate during growth of S. aureus, even if glucose was added to the medium. The growth medium consisted essentially of amino acids and peptides and the incubation medium was PBG. Because the presence of Pi and glucose during growth still allowed a very large accumulation of FDP, the only remaining difference in the two media was the amino acid content. The basic question, then, was which amino acids acted during growth to prevent the accumulation of FDP, or, expressed differently, which amino acids provided the proper intracellular environment that did not allow the accumulation of FDP. (This raised the question of the possible existence of an intermediate cellular state in which the cells were not growing but also not accumulating FDP.) Accordingly, various amino acids were added to the PBG incubation medium to determine their effects on the accumulation of FDP. The results of several experiments (Tables 21-26) indicated that cysteine alone could significantly inhibit, and, at sufficiently high concentration, completely prevent, the accumulation of FDP.

A number of experiments in which S. aureus was incubated in PBG with various concentrations of cysteine-HCl (Tables 24-26) resulted in

several conclusions regarding the effects of this amino acid on non-growing S. aureus. Cysteine caused a decrease in (i) the percentage of glucose catabolized by the HMP pathway, (ii) the actual amount of glucose utilized by this pathway, and (iii) the total glucose utilized. This indicates that cysteine may have acted at two levels, one, on the HMP pathway directly (probably on the oxidative portion which was measured in the experiments), and two, on the transport of glucose, which resulted in a decrease in the total glucose consumed. These experiments again illustrate the apparent variability of the HMP pathway relative to the EM pathway. During incubation of cells in the presence of 100 mM cysteine-HCl, the HMP pathway was almost completely eliminated, while the EM pathway was reduced by a maximum of 53% (Table 25, part C) and 55% (Table 27, part A), both in the presence of only 10 mM cysteine.

The apparent effect of cysteine on the catabolism of glucose via the HMP pathway (Tables 24-B, 25-C, and 26) suggested that the amino acid may have affected one of the enzymes in the oxidative portion of the pathway. Such an effect by cysteine would suggest that the enzymes possessed sulfhydryl groups that were involved in activity, and it was shown for 6PGD from both Bacillus stearothermophilus (304) and E. coli (318) that p-chloromercuribenzoate caused inactivation of the enzyme, indicating a possible role for sulfhydryl groups.

The fourth effect of cysteine on non-growing S. aureus was a large reduction in the activity of the TCA cycle. The activity of the TCA cycle usually decreased during incubation of S. aureus in PBG, shown by the radiorespirometric assays. These results suggested that one or more of the TCA cycle enzymes contained sulfhydryl groups which were involved

in the activity of the enzyme. Likely candidates for such potential effects of cysteine are the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, both of which contain dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase, which are involved in the reduction and oxidation of lipoic acid.

The fifth effect of cysteine, and the most significant for this investigation, was the inhibition of the accumulation of FDP during incubation of S. aureus. The discussion of this effect of cysteine will be presented later in conjunction with results of other experiments.

Another effect of cysteine during incubation was its apparent acceleration of the decrease in the level of NAD by as much as 2.5 fold. The level of NAD in S. aureus normally decreased during incubation in PBG. This was attributed to actual breakdown of NAD and not just conversion to NADH (R. Hoo, 1968. Levels of nicotinamide adenine nucleotides in metabolically altered staphylocci. M.S. Thesis, Loyola Univ. of Chicago).

What effect might such a decrease in NAD levels have on the glucose catabolic pathways? There are three enzymes, two in the HMP pathway and one in the EM pathway, that are points for potential regulation by NAD. In the HMP pathway of several organisms, G6PD and 6PGD were shown to consist of isozymes utilizing NAD and/or NADP. In S. aureus, Montiel et al. (200) found such isozymes of both G6PD and 6PGD. The levels of NAD required for activity of these enzymes in vitro was about 10 times greater than the required level of NADP. This 10:1 ratio of NAD: NADP is also the approximate ratio of these coenzymes in S. aureus. Therefore, it was reasonable to postulate that a reduction in the level of NAD would

result in lowered activity of the HMP pathway. In the EM pathway, the enzyme GAPD uses NAD as coenzyme, and thus appears to be a point for potential control, which would not necessarily be allosteric but may occur through a possible decrease below the level required for maximal activity of the enzyme. However, the pathways estimations indicated that the decrease in glucose utilization by the EM pathway was usually much smaller than that in the HMP pathway. Furthermore, examination of part A of Tables 24-26 shows that the extreme levels of FDP existed in cells in which the amount of glucose catabolized by the EM pathway differed by only a few percent.

The seventh effect of cysteine was an inhibition of the increase in the level of ATP in the non-growing cells (Table 23, part D, Table 27, part A, and Table 28, part B). There were several sites where cysteine could have had such an effect on ATP. These include (i) oxidative phosphorylation, (ii) the transport of Pi into the cells, and (iii) the transport of glucose into the cells. The nature of Pi uptake was studied by Mitchell (194), who found evidence for the transport of Pi into S. aureus, and that the alkylating agent, N-ethylmaleimide, inhibited Pi uptake, although iodoacetate had no effect (195). Such an effect by an alkylating agent suggests that sulfhydryl groups were involved in some aspect of Pi transport. Therefore, it is possible that a sulfhydryl reagent, such as cysteine, might also have some inhibitory effect on Pi transport.

The decreased utilization of glucose in the presence of cysteine, as determined in the pathways estimations and during a simulation of the pathways estimation (Table 32), suggested that cysteine may have had

some direct effect on the transport of glucose into the cells. This would have lowered the total glucose utilization, which did occur, but as indicated in Tables 24-26, the HMP pathway was considerably more affected than the EM pathway. However, as mentioned previously, the amount of glucose utilized after incubation of S. aureus in the presence of cysteine was only slightly reduced (Tables 24-26) and measurement of glucose utilization during the pathways estimation in the presence of cysteine (Table 33) showed very little effect of cysteine on glucose utilization. Therefore, any effect of cysteine on glucose transport into S. aureus was slight and most certainly did not significantly affect the levels of FDP. The studies on the effects of cysteine on the uptake of 2-deoxyglucose (Fig. 5 and 6) seemed to support this conclusion.

The eighth effect of cysteine was an apparent decrease in viability of the cells after incubation. These preliminary results were not conclusive and will not be discussed.

Throughout the greater part of this investigation, the cysteine used had been the hydrochloride form, which, when used at concentrations of 30 and 100 mM, even in 50 mM Pi buffer, required neutralization to pH 7. This was accomplished with KOH. However, neutralization of 30 or 100 mM cysteine-HCl required about 30 or 100 mM KOH, resulting in incubation media that were also 30 or 100 mM in KCl. Recalling data from Table 15, the effect of growth and incubation of cells in the presence of NaCl was a reduction in the accumulation of FDP. Longer exposure to NaCl resulted in greater reduction in the observed level of FDP. Therefore, it seemed reasonable that incubation of cells with KCl would have a similar effect. This was supported by data in Tables 16,

part B and 28, part B. Incubation of S. aureus with cysteine-HCl resulted in a lower accumulation of FDP than incubation with cysteine free base. The presence of 100 mM KCl apparently exerted effects similar to those of NaCl. Such effects of NaCl and KCl on FDP levels were presumed to be caused by the effects of the salts on the permeability of the staphylococcal cell wall and/or cytoplasmic membrane.

A comparison of cysteine with two other reducing agents, 2-mercaptoethanol (2ME) and dithiothreitol (DTT) (Table 27), indicated that only 2ME resembled cysteine in its effects on both pathways and intermediates. The pattern of change in the levels of FDP, NAD and ATP in the presence of 2ME were similar to those caused by cysteine. However, the levels of 2ME required for these effects were 20- to 50-fold higher than those of cysteine. This difference may be attributed to lower permeability of 2-ME compared to cysteine, or that 2ME is not an amino acid and thus may not have the same effector properties of cysteine. The smaller effects of DTT may have been caused by the presence of two reducing equivalents (compared to one each for cysteine and 2ME), which may have inhibited entry of DTT into the cells. That at least one other, non-physiological reducing agent, 2ME, had effects similar to those of cysteine suggested that the role of cysteine was primarily as a reducing agent in the non-growing cells. This further suggested that cysteine may have acted at more than one site in the cells, and that the effects on FDP may have resulted from a convergence of several of these effects.

Accumulations of FDP have been observed in other organisms, each caused by a unique set of conditions. Holzer (124, 125) observed an accumulation of FDP to about 2 μmol/g wet wt (0.4 μmol/g dry wt) in

yeast cells starved aerobically in the presence of glucose. He proposed a mechanism whereby a lack of acetaldehyde, caused by its diffusion into the medium, prevented reoxidation of the NADH produced by the GAPD reaction. Additions of acetaldehyde to the incubation medium reduced the FDP accumulation. Mizushima and Kitahara (197) observed FDP levels up to 17 mM in Lactobacillus plantarum after cells were incubated in Pi-buffered glucose. They attributed this accumulation of FDP to a combination of the Harden-Young effect and the inability of non-growing cells to use ATP for biosynthetic reactions. Böck and Neidhardt (21) found FDP levels of 145 mμmol/mg protein (about 80 μmol of FDP/g dry wt) in a temperature-sensitive FDP aldolase mutant of E. coli growing in the presence of glucose. Yamada and Carlsson (329) found that Streptococcus bovis contained up to 55 nmol of FDP/mg dry wt during anaerobic growth with excess glucose.

Consideration of the levels of seven of the EM pathway intermediates in 17-h growing cells indicated that all except PEP were at a concentration less than 1.0 μmol/g dry wt. These were the levels in cells growing in an amino acid medium with no added carbohydrate, i.e., conditions where the EM pathway operated gluconeogenically. However, even when the cells were grown in the presence of glucose, the levels of the intermediates were not significantly different. (Such levels were indicated at zero time in the tables.) The only EM pathway intermediate that was consistently above 1 μmol/g dry wt at zero time was PEP. Low levels of most EM pathway intermediates were found by Lowry et al. (179) and by Moses and Sharp (204) in E. coli grown with a number of carbon sources.

The level of NAD in growing S. aureus was generally 3-5 μmol/g. This may be compared with the results of London and Knight (176), who surveyed a number of microorganisms and divided them into three groups on the basis of their NAD content. They found that strict anaerobes and lactic acid bacteria had NAD concentrations above 4.5 μmol/g dry wt and facultative anaerobes had concentrations of 1.0-3.0 μmol of NAD/g. Strict aerobes had less than 1 μmol of NAD/g. They also found that addition of nicotinic acid (1.5 mg/l) to a minimal medium in which E. coli was growing resulted in a 4-fold increase in NAD (from 2.4 to 9.4 μmol/g) and a doubling of NADP (from 0.24 to 0.45 μmol/g). A similar occurrence was observed in S. aureus by Blumenthal (17), who added 2 μg of niacin/ml to a thiamine-supplemented (2 μg/ml) VFC growth medium and found a 19-fold increase of NAD (from 0.3 to 5.7 μmol/g) and a 3-fold increase of NADP (from 0.2 to 0.6 μmol/g). These data indicate that S. aureus behaved as a typical facultative anaerobe with regard to its levels of NAD according to the grouping of London and Knight (176). That the NAD levels in S. aureus were generally 8-10 times higher than those of NADP may partially reflect the greater utilization of the EM pathway rather than the HMP pathway for carbohydrate catabolism.

The levels of intermediates in non-growing S. aureus contrasted sharply with those in growing cells. As shown in Fig. 1 and in Table 4, the levels of G6P, FDP, DHAP, 6PG, and ATP increased over as long as a 7-h incubation period.

Because the greatest change occurred in the level of FDP, interest centered on determining the mechanism of such an unusual accumulation. The second major finding of this investigation was the apparent locali-

zation of the control of the FDP accumulation at GAPD. However, it is appropriate to briefly examine the other enzymes that may have been involved in the accumulation of FDP. These are phosphofructokinase (PFK), FDPase, FDP aldolase, triosephosphate isomerase (TPI), and glyceraldehyde-3-phosphate dehydrogenase (GAPD). An accumulation of FDP could have been caused by stimulation of PFK, but then no increase in the levels of G6P or F6P would have occurred. As shown in Fig. 2 and in Tables 4 and 5, increases in the levels of G6P and F6P did occur during incubation of S. aureus. FDPase was present in S. aureus, as demonstrated in partially purified crude extracts (Results). This enzyme would be essential during growth of cells under conditions of gluconeogenesis, such as in the amino acid media used here. However, FDPase would probably not be an important factor during incubation, partly because of the relatively large concentration of glucose, which would promote glycolysis.

An inhibition of FDP aldolase would cause an accumulation of FDP, as was demonstrated by Böck and Neidhardt (21) in a mutant of E. coli with a temperature-sensitive FDP aldolase. But then no increase in the levels of DHAP or GAP would be expected. However, as shown in several tables, the levels of all three intermediates increased during incubation of S. aureus. TPI interconverts DHAP and GAP, the products of FDP aldolase. Because FDP yields equal amounts of these triose phosphates, an inhibition of TPI would cause DHAP to accumulate, but not GAP because of its further metabolism in the EM pathway. Some FDP would probably accumulate because of the equilibrium of FDP aldolase, and the extent of the FDP accumulation would depend on the rate of FDP formation, the rate of FDP breakdown by FDP aldolase, and also on any further metabolism of DHAP. Rose and Rose (246) indicated that DHAP was more important than

GAP in the mechanism of the FDP aldolase reaction, so it is possible that high levels of DHAP caused by a TPI inhibition could cause considerable accumulation of FDP. However, because both triose phosphates accumulated during incubation, an inhibition of TPI was unlikely. Noltmann (214) referred to TPI as the "fastest" enzyme in intermediary metabolism, with a molecular activity of 500,000-1,000,000 per min at 25 C. This would suggest that a virtually complete inhibition of this enzyme would be required to produce any effects in vivo.

GAPD remained as a site for potential inhibition during incubation, as suggested by several lines of evidence. (i) The results of incubation in the presence of arsenate (Table 14) indicated a reduced accumulation of FDP and DHAP, with a larger accumulation of pyruvate. The probable role of arsenate in these cells was to replace Pi in the GAPD reaction, forming 3-phosphoglyceroyl 1-arsenate, which spontaneously breaks down to form 3-phosphoglycerate and arsenate, thus by-passing the phosphoglycerate kinase reaction. Note that this reasoning suggested a role for phosphoglycerate kinase (PGK) in the accumulation of FDP, as was suggested by Burton (30) and by Rose and Rose (246). During incubation, high levels of ATP and correspondingly low levels of ADP could have inhibited PGK and caused some accumulation of 1,3-diphosphoglycerate and GAP, resulting in an accumulation of FDP. In the presence of arsenate, PGK was probably by-passed, so that 1,3-diphosphoglycerate did not accumulate. Although these results may have suggested a possible role for PGK in the accumulation of FDP, the high activity of this enzyme in S. aureus (15), a lack of known allosteric effectors, and a reaction depending only on the concentration of Mg^{+2} and ADP (182, 246) suggested

that the role of PGK in the accumulation of FDP in non-growing S. aureus may be minimal.

(ii) The probable involvement of GAPD in the accumulation of FDP suggested the use of iodoacetic acid (IAA), an alkylating agent and enzyme inhibitor to which GAPD is especially sensitive (44, 247). The effects of incubation of S. aureus in PBG plus IAA are shown in Table 28. In the presence of IAA, the levels of FDP and DHAP were much higher than in the controls without IAA. Several items may be noted from these results: (a) Both FDP and DHAP increased significantly in the presence of IAA. The apparent inhibition of GAPD by IAA caused little or no accumulation of GAP, the substrate of the enzyme in the glycolytic direction, yet FDP and DHAP accumulated to the highest levels observed in this investigation. (b) The level of PEP decreased to values < 1.0, indicating that the flow of carbon along this latter half of the EM pathway was severely curtailed. (c) The level of ATP decreased during incubation of the non-growing cells in the presence of IAA. Apparently, the inhibition of GAPD was sufficient to ultimately inhibit the rate of oxidative phosphorylation, hence, ATP levels did not increase as usual during incubation.

As mentioned previously, during incubation of non-growing cells in the presence of IAA, the level of ATP actually decreased during incubation (Table 28). If the production of ATP was reduced or completely inhibited in the presence of IAA, what was the fate of the ATP that was lost during incubation? Apparently, this ATP was consumed by PFK during synthesis of the much greater amount of FDP formed in the presence of IAA. The role of ATP in the accumulation of FDP apparently was significant and will be discussed in more detail subsequently.

(iii) The third line of evidence regarding the role of GAPD in the accumulation of FDP involves the enzyme FDP aldolase. As mentioned above, during incubation with IAA, the level of GAP remained essentially constant, while FDP and DHAP approximately doubled. This suggested an indirect role for FDP aldolase in the accumulation of FDP and DHAP. FDP aldolase has an equilibrium constant of about 10^{-4}, hence, the reaction is readily reversible. The ratios of FDP:DHAP:GAP may be 1:1:1 or 1000:10:10 and the reaction is still in equilibrium (246). As shown in a number of tables from this investigation, the ratio of FDP:DHAP:GAP in growing cells (after 17 h) was approximately 1:1:1. After incubation, the levels of FDP were generally 60-100 μmol/g, occasionally up to 200 μmol/g. DHAP ranged over 2-10 μmol/g and GAP $<$1.0-2.0 μmol/g. These ratios thus resemble the theoretically possible 1000:10:10 ratio, but since these levels were obtained from growing cells, the equilibrium of TPI affected the results. The equilibrium of TPI results in a DHAP:GAP ratio of 22:1. Therefore, a higher level of DHAP than GAP would be expected in vivo, and such were the results (Table 28). These theoretical considerations on the equilibrium of FDP aldolase support the notion that GAPD was a major site of inhibition during incubation of S. aureus in PBG. The presence of IAA during incubation caused much higher levels of FDP and DHAP, but the ratio of FDP:DHAP:GAP remained within the theoretical limits of the FDP aldolase equilibrium.

(iv) The fourth and final line of evidence on the role of GAPD in the accumulation of FDP involves cysteine and its effects in vivo and on GAPD in vitro. The effects of cysteine during incubation of whole cells were shown by the series of experiments represented by Tables 21-26.

After the initial observation that a mixture of thirteen amino acids inhibited the accumulation of FDP, the number was reduced to one, cysteine, which at sufficiently high concentration completely inhibited the accumulation of FDP. It was then demonstrated that one other sulfhydryl reagent, 2ME, at higher concentration (Table 27), had effects similar to those of cysteine.

Cysteine prevented the accumulation of FDP, and as important was the observation that cysteine (in the presence of arginine and glutamine) was able to reverse the FDP accumulation, as shown in Table 30 and Fig. 4. This suggested that cysteine, and presumably 2ME, were acting as sulfhydryl (reducing) agents, rather than as allosteric effectors.

That the level of FDP increased after removal of cysteine and decreased after addition of cysteine over a period of 4 h suggested that the intracellular population of enzymes remained essentially constant and that there was no major catabolism of enzymes, especially of those in the EM and HMP pathways. Furthermore, the PBG incubation medium, lacking a nitrogen source, would not allow protein synthesis. To ensure that no de novo protein synthesis occurred using amino acids from the possible breakdown of existing enzymes, S. aureus was incubated in the presence of chloramphenicol. As shown in Table 31, chloramphenicol had no effect on the accumulation of FDP in PBG, nor did it alter the effect of cysteine in inhibiting the accumulation of FDP. Therefore, the accumulation of FDP resulted not from the synthesis of enzymes with altered regulatory properties, but from inhibition of those enzymes in the relatively enzymologically-stable intracellular environment of the non-growing cells.

The major conclusion from the fourth line of evidence was that GAPD from S. aureus required a reduced environment for optimum activity, and that such an environment was provided by cysteine and 2ME during incubation. This conclusion was supported by the results of the in vitro assay of GAPD (Table 40), which indicated that there was little or no activity of GAPD in the absence of cysteine. As might have been predicted from the in vivo results, the in vitro assays of GAPD showed that 2ME at higher concentration replaced cysteine in activating the enzyme (Table 41). In contrast to the in vivo results, however, dithiothreitol, at concentrations lower than cysteine, was also effective in activating GAPD.

The major observations on GAPD from S. aureus in this investigation were: (i) A reducing agent was required for activity. The requirement for a reducing agent in assays of GAPD is a widely occurring phenomenon. Cysteine and 2ME were used at concentrations ranging from 3-13 mM in assays of GAPD from organisms ranging from E. coli to rabbit (44, 67, 75, 122, 129, 162, 168, 190, 217, 301). DTT was effective at lower concentrations on the spinach (238) and rat muscle enzymes (327), and Heilmann and Pfleiderer (110) obtained full activity of their yeast GAPD preparation after it was preincubated in 0.5 mM DTT. Thus, it is evident that a reducing agent was essential for activity of GAPD, which may be partially explained by the presence of a cysteine residue at the active site and its participation in the reaction (246, 302). Rose and Rose (246) mentioned that the relationship of the sulfhydryl groups at the active site of GAPD to the redox state of the environment around the enzyme may have regulatory significance. (ii) GAPD from S. aureus was very sensitive to inhibition by IAA, demonstrated both in vitro and in

vivo (Table 28). The inhibition by IAA in the in vitro assay could be neither prevented nor overcome by cysteine. This indicated that the S. aureus GAPD was similar in this respect to the enzyme from other sources (44, 302).

Briefly, the major observations on FDP aldolase were the following: (i) Some of the activity appeared to be inhibited by EDTA (Table 37). Inhibition of FDP aldolase by EDTA is a characteristic of class II aldolases, found in bacteria, fungi, and blue-green algae (130, 201). However, the presence of non-EDTA sensitive FDP aldolase activity suggested that a class I aldolase also occurred in S. aureus. The simultaneous existence of both class I and class II aldolases was observed in Euglena and Chlamydomonas (250), and in Micrococcus (Peptococcus) aerogenes (169). (ii) A majority of the activity of the S. aureus FDP aldolase occurred in the supernatant fluid after precipitation with 100% ammonium sulfate (Table 38). This, combined with the observation that one band of FDP aldolase activity migrated with the marker dye during gel electrophoresis, suggested that the S. aureus FDP aldolase had a relatively low molecular weight. The class I aldolase found in Micrococcus aerogenes was unique in consisting of a single polypeptide of molecular weight 33,000 (170).

The third major finding of this investigation was the apparent lack of effect of the large intracellular accumulation of FDP on the extent of glucose catabolism via the HMP pathway. Therefore, it was desirable to not only estimate the pathways in the presence of high and low FDP levels, but also to actively vary the HMP pathway to observe any possible relationship between pathway activity and the accumulation of FDP. The niacin analog 6AN was used to vary the activity of the HMP pathway.

As shown in Tables 5 and 6, incubation of S. aureus in PBG plus increasing concentrations of 6AN resulted primarily in greater accumulations of 6PG and lower activity of the HMP pathway. The high concentrations of 6AN during incubation had essentially no effect on the EM pathway intermediates.

The effects of 6AN on intermediates and pathways are shown in Tables 6-9. Again, increasing concentrations of 6AN during incubation resulted in higher accumulations of 6PG and activity of the HMP pathway that was inversely related to the level of 6PG. It was consistently observed that FDP accumulated to very high levels, which were similar within each experiment, but which were not apparently related to the concentration of 6AN, nor to the level of activity of the HMP pathway. The results of Tables 6-9 may be summarized: (i) FDP accumulated during incubation of S. aureus in PBG; (ii) 6PG accumulated during incubation of cells in PBG + 6AN; (iii) the presence of 6AN during incubation caused a reduction in the activity of the HMP pathway, which was manifested as decreases in the percentage and amount of glucose catabolized via this pathway; (iv) the accumulation of FDP apparently was independent of 6AN and of the activity of the HMP pathway; (v) although the percentage of the EM pathway increased as a consequence of the decreased percentage of the HMP pathway, the actual amount of glucose catabolized by the EM pathway remained fairly constant under a variety of incubation conditions within each experiment, even in the presence of very high FDP levels. It was also observed that (vi) glucose, and to a lesser extent 6AN, accelerated the reduction in the level of NAD during incubation. Also apparent from Tables 8 and 9 is that no accumulation of intermediates occurred in the absence of glucose.

These observations indicated that the activity of the HMP pathway could be varied independently of the intracellular level of FDP. Therefore, under the conditions used here, FDP apparently did not affect the activity of the HMP pathway.

That 6AN affects the HMP pathway is well established. Johnson and McColl (139) first described the effect of 6AN as an antagonist of niacin in the conversion of niacin to NAD. Dietrich et al. (54) treated mice with 6AN and isolated the 6AN analogs of NAD and NADP. Herken and Lange (117) injected rats with 6AN and observed large accumulations of 6PG. Köhler et al. (158) and Lange and Proft (165) observed a strong inhibition of 6PGD from rat tissues by the 6AN analog of NADP. Ofori-Nkansah and von Bruchhausen (216) observed a 70-fold increase in the level of 6PG and a 3-4-fold increase of G6P in Yoshida ascites tumor cells incubated in the presence of 6AN. The mode of action of 6AN in S. aureus is apparently the same, as was first demonstrated by Hoo (126), who found an accumulation of 6PG during incubation of S. aureus in PBG plus 6AN.

Because the radiorespirometric method employed the evolution of labeled CO_2 from 6PG, only the oxidative portion of the HMP pathway was actually measured. Therefore, the very high intracellular levels of FDP had no apparent effect on the oxidative portion of the HMP pathway. In contrast to these negative in vivo results were the results of the in vitro assays of 6PGD. As shown in Table 42, 5 mM FDP inhibited the activity of 6PGD, whether FDP was present initially or added during the reaction. This agreed with the results of Brown and Wittenberger (24), who found that 10 mM FDP inhibited 6PGD from Streptococcus faecalis and from all animal and microbial sources of enzyme tested. This illustrates

that in vivo and in vitro data will not always corroborate one another and that it is not always possible to correctly project from one to the other.

Brown and Wittenberger later demonstrated (26) that S. faecalis grown in gluconate possessed both NAD- and NADP-linked 6PGD, but only the NADP-linked enzyme was inhibited by FDP, and only the NAD-linked enzyme was inhibited by ATP.

Previous work in this laboratory showed the existence of both NAD- and NADP-linked G6P and 6PG dehydrogenases in S. aureus (18). Only the NADP-linked enzymes were inhibited about 50-60% by FDP, as demonstrated in a 40-70% ammonium sulfate fraction of streptomycin sulfate-treated S. aureus extract (Montiel and Blumenthal, unpublished observations).

Also in Table 42, it was shown that cysteine could partially prevent the inhibition of 6PGD by FDP, and that cysteine could partially overcome such inhibition. Brown and Wittenberger found a similar protective effect of 2-mercaptoethanol on 6PGD from S. faecalis (24). These results may explain the apparent lack of an in vivo effect of the high FDP levels on the HMP pathway. Cysteine or other metabolites in vivo could possibly prevent the inhibition of 6PGD by FDP demonstrated in vitro.

Brown and Wittenberger (24) found an in vitro inhibition of 6PGD by FDP in S. faecalis and then suggested a possible in vivo mechanism for the regulation of the HMP pathway. Although they used anaerobic conditions for growth of their cells and for measurement of $^{14}CO_2$ evolution from labeled glucose and gluconate, they did not estimate actual HMP pathway participation nor did they measure the intracellular levels

of FDP under conditions where they might have been expected to change, hence possibly having an effect on the HMP pathway. In fact, it seems that most of the speculation in the literature concerning metabolic regulation has been based on in vitro observations of enzyme activities as modified by potential effectors in not entirely natural circumstances, which is generally not possible under in vitro conditions. Apparently, little consideration has been given to estimation of pathways in vivo or to correlation of in vitro and in vivo results. For these reasons, the system used here, of pathways estimations correlated with measurement of intermediate levels, is perhaps superior to in vitro assays only, and provides stronger evidence for what may be the actual situation. This approach allowed some comparison of data obtained on isolated and partially purified enzymes with data derived from the in vivo estimations of the pathways in which the enzymes were involved. Again, it must be emphasized that even though such a comparison between the in vivo and in vitro situations can be made, it may not necessarily result in successful correlation between the two, as demonstrated by the effects of FDP on 6PGD in vitro and an apparent lack of effect in vivo. Such lack of correlation between the two approaches was discussed by Fraenkel and Vinopal (85), by Sanwal (253), and by Srere (273).

Recent attempts to solve the problem of in vitro vs. in vivo enzyme activities have used "permeabilized cells," i.e., cells treated with toluene followed by freezing and thawing to make the cell membrane freely permeable to small molecules and enzymes. This method has the advantage of resembling the physiological condition of the enzyme with respect to enzyme concentration and interactions with other cell constituents.

Reeves and Sols (240) examined the activity of PFK in E. coli treated with toluene. Kornberg and Malcovati (159) examined PK in permeabilized E. coli.

Studies by Weitzman on citrate synthase from Klebsiella aerogenes (316) and from yeast (317), indicated that the properties of enzymes observed in situ quite closely resembled those from the in vitro assays. This approach may be useful in further studies of regulatory enzymes and may serve as a useful bridge between completely in vitro and in vivo assays.

Because the glucose catabolic pathways in S. aureus were estimated under conditions similar to those of incubation, it was desirable to determine the possible effects of the pathways estimations on FDP levels. In one experiment scaled up from that used in the pathways estimations (Fig. 2), all assayed intermediates, FDP included, followed the pattern observed consistently in the standard incubation experiments. However, the accumulation of FDP underwent a spontaneous reversal in the pathways simulation, probably because of the lower initial glucose concentration (one-ninth of that used in the standard incubation experiments). In the second experiment designed to simulate the pathways estimation conditions (Table 32), the results obtained from cells incubated in the pathways estimation medium were comparable to those obtained previously (Fig. 2). The addition of cysteine to the incubation medium resulted in a much lower accumulation of FDP and an increase in the breakdown of NAD (Table 32). The percent of glucose used in the presence of cysteine was only about 67% that used in the absence of cysteine. A reduction of glucose utilization by 33% can not be the only explanation for a 94% reduction in FDP accumulation. This initially suggested an effect of cysteine on

the uptake of glucose, but the much lower accumulation of FDP suggested that cysteine was acting at one or more other sites during incubation of non-growing cells. As indicated previously (Tables 17, 23, 27, and 28), cysteine appeared to inhibit the accumulation of ATP during incubation. This will be considered in more detail subsequently.

Returning to the role of Pi in the accumulation of FDP, the data in Table 12, part B, indicated that growth of S. aureus in VFC supplemented with Pi resulted in lower accumulations of ATP during incubation with increasing Pi concentrations than incubation in non- Pi buffer without added Pi. When cells were grown in VFC without added Pi and incubated in non-Pi buffer, the level of ATP decreased and FDP did not accumulate. Data in Table 20, part B, indicated that growth of cells in VFC followed by incubation in non-Pi buffers resulted in smaller increases in the levels of both ATP and FDP. These data strongly suggested a relationship between the levels of ATP and FDP in the non-growing cells. The parallel accumulations of both ATP and FDP depended on the presence of Pi during incubation (Tables 12-B, 20-B), and an accumulation of ATP was necessary for the accumulation of FDP (Table 27).

The possible dependence of the FDP accumulation on ATP suggested that inhibition of ATP synthesis should lower the FDP accumulation. This was tested in two types of experiments. Incubation of cells under anaerobic conditions, which inhibited ATP production by oxidative phosphorylation, resulted in lower accumulations of FDP (Table 34).

The second type of experiment involved the use of an uncoupling agent, carbonyl cyanide m-chlorophenylhydrazone (CCCP). The effects of CCCP were initially demonstrated by Heytler (120). Incubation of S.

aureus in PBG plus CCCP resulted in almost complete inhibition of the accumulation of ATP and much smaller accumulations of FDP (Table 29). Apparently, the accumulation of ATP was directly inhibited by CCCP, resulting in a significant inhibition of the accumulation of FDP. This provided evidence for the dependence of the FDP accumulation directly on the accumulation of ATP in the non-growing cells. An explanation for the accumulation of ATP in non-growing cells and its relation to the accumulation of FDP will now be presented in a discussion of a possible mechanism for the accumulation of FDP.

The levels of FDP attained during incubation were as high as 200 μmol/g dry wt. This is equivalent to an intracellular concentration of 44 mM, based on the assumption that the dry weight was 20% of the wet weight and that the cell density was 1.1 g/ml (204). The extent of accumulation of FDP was directly affected by glucose and Pi during incubation of non-growing cells. Incubation of S. aureus in non-Pi buffers resulted in lower accumulations of FDP than when cells were incubated in Pi buffer. Incubation in PBG not only caused a very large accumulation of FDP, but also resulted in a substantial increase in the level of ATP. The changes in ATP tended to parallel those of FDP, as when cells were incubated in non-Pi vs. Pi buffers, in the presence of reducing agents (cysteine and 2ME), and especially in the presence of an uncoupling agent of oxidative phosphorylation (CCCP). These observations suggested that the accumulation of FDP depended on that of ATP.

The role of Pi during incubation was severalfold. It served as the buffer for the system, it acted as a salt, and perhaps most importantly, it was a source of Pi for cells that had been grown without added

Pi in an apparently Pi-deficient medium. Thus, after growth, the cells were confronted with a much higher concentration of Pi, under non-growing conditions. That Pi was shown to be transported into S. aureus (194, 195) suggested that there was a limit to the rate of uptake of Pi. Because the Pi transport system of E. coli involves a binding protein (191), saturation of such a system was possible, as was demonstrated for Neurospora at about 2 mM Pi (178).

After growth of S. aureus in the presence of 50 mM Pi, the cells did not accumulate as much FDP or ATP during incubation as did cells grown without added Pi. The initial explanation for this might be that cells grown with Pi had a larger Pi pool (Pi plus ATP, ADP, and AMP). However, the zero-time level of ATP in cells grown with Pi was not significantly higher than that in cells grown without added Pi (Table 12). A study by Forrest (78) showed that the presence of Pi during growth of Streptococcus faecalis had only a small effect on ATP levels: 6.75 μg/mg dry wt during exponential growth without Pi and 5.1 μg/mg in the presence of 0.1 M Pi. Other studies on E. coli (86), Neurospora (270), and Chromatium (193) indicated that the levels of ATP, ADP, and AMP remained essentially constant in widely differing growth media.

Another possible explanation for the lower accumulation of FDP and ATP after growth with Pi involves the phenomenon of Pi uptake. Perhaps growth with Pi resulted in a lower rate of transport of Pi into the cells during incubation. Such was observed by Medveczky (192), who found that after growth without Pi, E. coli took up Pi in a biphasic manner, i.e., rapidly for 1 min followed by a slower rate for 9 min. In contrast, cells grown with 50 mM Pi had a rapid initial uptake, which decreased

to a much lower rate after 10 min.

If Pi was a factor limiting the growth of the organisms, then addition of Pi to the non-growing cells and the resulting accumulations of FDP and ATP suggested that there was a lack of control in glucose and/or Pi utilization. As was observed, the presence of both glucose and Pi seemed to act synergistically to stimulate growth of S. aureus. This follows from the observation of Tanzer et al. (288) that accumulation of Pi in non-growing streptococci was energy-dependent, and from that of Goodman and Rothstein (99), who found that glucose stimulated the uptake of Pi in non-growing Saccharomyces cerevisiae.

A possible loss of metabolic control in non-growing S. aureus may have occurred at two metabolic sites in the cells. One site was GAPD in the EM pathway, the partial inhibition of which resulted in accumulation of FDP. Incubation of cells in the absence of Pi resulted in a smaller accumulation of FDP and correspondingly smaller accumulations of ATP (Tables 12 and 20). Inhibition of GAPD would result in an accumulation of FDP and of the triose phosphates (315).

During incubation, a second site in the non-growing cells may have been the rapid uptake of Pi and its incorporation into ATP. During incubation of these relatively Pi-deficient cells, Pi may have entered their metabolism by two possible routes. One, via the GAPD reaction, would have been insignificant compared to the potential Pi incorporation by the second, the formation of ATP by oxidative phosphorylation in the highly aerobic environment present during incubation. In such non-growing cells, ATP could not be used for biosynthetic reactions (43, 197), hence, it accumulated. Because about 80% of the glucose was being metabolized

via the EM pathway (after 3 h of incubation), the excess ATP was used by PFK to form FDP, which subsequently accumulated. The excess ATP significantly contributed to the FDP accumulation initiated by the reduced activity of GAPD.

Commoner (43) discussed the role of ATP in the regulation of biosynthesis, indicating that since ATP was a product of oxidative metabolism, utilization of ATP and the corresponding increase of ADP, during protein synthesis, for example, would cause positive feedback to increase the rate of oxidative metabolism and thereby replenish the supply of ATP. In non-growing cells, feedback would not be operative. Forrest and Walker (79) observed large increases of ATP (up to 6 μg/mg, or to about 10 μmol/g dry wt) in Streptococcus faecalis incubated anaerobically in Pi-buffered glucose, and suggested that there was no feedback control of glycolysis in these cells. In a study of the effect of pantothenate starvation on the growth of Zymomonas, Belaich et al. (9) concluded that anabolic processes did not control catabolism, and that in contrast to biosynthesis, catabolic and energy-generating reactions were less controlled.

The large concomitant increases of FDP and ATP in non-growing S. aureus incubating in PBG, apparently caused by a partial inhibition of GAPD in the EM pathway and a simultaneous rapid and uncontrolled formation of ATP, suggests that the PFK reaction served as a metabolic outlet for the excess ATP. The high levels of ATP apparently did not inhibit PFK. Such an ATP-insensitive PFK was described by Thomas et al. (293) in aerobically-grown E. coli. Lazdunski and Belaich (167) also found that ATP accumulated during growth limitation of Zymomonas. In S. aureus under the conditions used here, FDP may have been a reservoir for excess

Pi derived from ATP.

Incubation of S. aureus in PBG plus the sulfhydryl reagents cysteine and 2ME resulted in lower accumulations of both FDP and ATP (Tables 16, 23, 27, and 28). These effects of cysteine and of 2ME suggested that they were acting at two sites in the cells. Based on the requirement for a reducing agent for in vitro activity, the enzyme GAPD was apparently one of the two possible sites. Rose and Rose (246) suggested that GAPD was sensitive to the oxidized or reduced state of the environment of the enzyme, based on the presence of cysteine at the active site.

The second site of influence by the sulfhydryl reagents may have been on the uptake of glucose and/or Pi. Data in Tables 25-27 showed that incubation with cysteine resulted in a decrease of glucose utilization, but the major effect was observed in the HMP pathway. Data in Table 32 showed that incubation of cells in PBG plus cysteine resulted in utilization of 63% as much glucose as during incubation in PBG without cysteine. Incubation of S. aureus in the presence of cysteine resulted in a decreased uptake of 2-deoxyglucose (2DG) (Fig. 5 and 6). Surprisingly, DTT caused a greater reduction in uptake of 2DG than did cysteine. However, a reduction of glucose uptake by 30-40% would not be sufficient to account for the much greater effect of cysteine on the accumulation of FDP. Concerning Pi, the work of Mitchell (194) showed that Pi was transported into S. aureus, and that the exchange of Pi across the membrane was inhibited by the sulfhydryl reagent N-ethylmaleimide (10 and 100 mM), but not by 100 mM IAA (195). In their studies of Pi transport in Bacillus cereus, Rosenberg et al. (249) found that 0.1 mM N-ethylmaleimide and 0.5 mM IAA inhibited Pi uptake by about 72%. The greater accumulation

of FDP in S. aureus in the presence of IAA indicated that there apparently was no significant inhibition of either glucose or Pi uptake. Harold and Baarda (106) observed that CCCP and other uncoupling agents inhibited energy-dependent Pi transport, with energy supplied by glucose. They suggested that the inhibition of transport could not have been caused by the uncoupling of oxidative phosphorylation, because the organism (Streptococcus faecalis) relied upon glycolysis for generation of ATP even under aerobic conditions. Incubation of S. aureus in the presence of CCCP caused a large reduction in the uptake of 2DG (Fig. 6). Perhaps the possible inhibition of Pi uptake by CCCP secondarily inhibited the uptake of 2DG.

In this investigation of the accumulation of FDP in non-growing S. aureus, attention has expanded from consideration of GAPD as a possible control point in the EM pathway mediated by cysteine to the possible effects of cysteine and other sulfhydryl reagents on the uptake of glucose and/or Pi. Consideration of these as separate phenomena was justified because Harold et al. (107) showed that GAPD was not involved in the uptake of Pi by Streptococcus faecalis. This was in contrast to the work of Goodman and Rothstein (99), who suggested that GAPD might be important in Pi uptake by yeast. This was based partly on the work of Prankerd and Altmann (237), who measured the relative specific activities of phosphorylated intermediates derived from ^{32}Pi in mammalian erythrocytes. They found high levels of labeled 2,3-diphosphoglycerate and suggested that the GAPD reaction was a major point of entry for Pi. However, their measurements were made over several hours, whereas Harold et al. (107) found that ATP, ADP, and the EM pathway intermediates G6P and FDP

were labeled from ^{32}Pi in 5 sec. But GAPD can not be eliminated from participating in the entry of Pi into cellular metabolism.

In conclusion, the extreme intracellular accumulation of FDP, to as high as 45 mM, in non-growing S. aureus was apparently caused by a combination of a partial inhibition of GAPD in the EM pathway and an uncontrolled formation of ATP resulting from incubation in PBG. Even in the presence of such high FDP levels, about 80% of the glucose was catabolized via the EM pathway, and there was no apparently significant inhibition of the HMP pathway. The accumulation of FDP was inhibited by the sulfhydryl reagents cysteine and 2ME and by an uncoupling agent, CCCP. The greatly increased accumulation of FDP during incubation with IAA plus the requirement for a reducing agent (cysteine, 2ME, or DTT) for in vitro activity implicated GAPD as the control point in the EM pathway directly involved in the accumulation of FDP. The large accumulation of ATP during incubation indicated that the incorporation of Pi into ATP was not controlled in the non-growing cells. The lack of feedback control of the formation of ATP coupled with the partial inhibition of GAPD apparently resulted in the accumulation of FDP. Therefore, the pathway of Pi during incubation was from the medium to ATP to FDP. The feedback control that would normally correct an imbalance between growth rates and ATP levels was inoperative in non-growing S. aureus.

SUMMARY

The two major pathways of glucose metabolism in Staphylococcus aureus are the Embden-Meyerhof (EM) and the hexosemonophosphate (HMP) pathways. Although relatively little is known about the mechanisms that regulate these pathways in growing cells, even less is known about their activity in non-growing cells. The problem is complicated by the uncertainty of whether the observations of the in vitro activities of enzymes accurately reflect the in vivo situation, especially regarding effectors of the enzymes.

The subject of this investigation was the control of glucose catabolism in non-growing S. aureus incubating in phosphate (Pi)-buffered glucose. The initial experiments were prompted by an observation that the EM intermediate fructose-1,6-diphosphate (FDP) inhibited the in vitro activity of the HMP enzyme 6-phosphogluconate dehydrogenase (6PGD) and the suggestion that FDP thus regulated the HMP pathway in a Streptococcus. Therefore, the problem was to determine the role of FDP in the regulation of the HMP pathway in S. aureus.

The three basic methods used were (1) incubation of S. aureus in Pi-buffered glucose plus various additions to determine their effects on FDP levels measured in phenol extracts of the cells, (2) estimation of the activity of the EM and HMP pathways by radiorespirometry before and after incubation, and (3) preparation of partially purified cell extracts for assay of enzyme activity.

The three major findings of this investigation were: (1) After growth of S. aureus in two vitamin-supplemented amino acid media, and incubation in Pi-buffered glucose, FDP accumulated from less than 1.0 to 87.3 $\pm$ 17.3 and to 116.1 $\pm$ 38.6 μmol/g.

FDP levels as high as 200 μmol/g (44 mM) were also observed. The accumulation was dependent on glucose and other carbohydrates, was augmented by Pi and iodoacetate, and was inhibited by cysteine, other reducing agents, and an uncoupling agent (CCCP) during incubation. The presence of glucose, Pi, and NaCl during growth and the absence of thiamine during growth inhibited the accumulation of FDP during subsequent incubation.

(2) The control point for the FDP accumulation appeared to be glyceraldehyde-3-phosphate dehydrogenase in the EM pathway, suggested by the effects of iodoacetate and cysteine in vivo, and by the requirement for cysteine for activity of the enzyme in vitro.

(3) The high intracellular levels of FDP apparently did not affect the in vivo activity of the HMP pathway measured by radiorespirometry, nor the activity of 6PGD in vivo because 6PG did not accumulate significantly, even in the presence of high levels of FDP. However, FDP did inhibit 6PGD in vitro. The presence of 6-aminonicotinamide during incubation caused a large accumulation of 6-phosphogluconate and inhibition of the HMP pathway, but did not significantly affect the accumulation of FDP.

The parallel changes in ATP and FDP, and the effects of cysteine and CCCP on ATP and FDP levels, suggested that FDP accumulated because of the combined effects of an uncontrolled production of ATP and a partial inhibition of glyceraldehyde-3-phosphate dehydrogenase during incubation of the non-growing cells in the presence of glucose and phosphate.

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APPROVAL SHEET

The dissertation submitted by Charles Frederick Huettner has been read and approved by the following committee:

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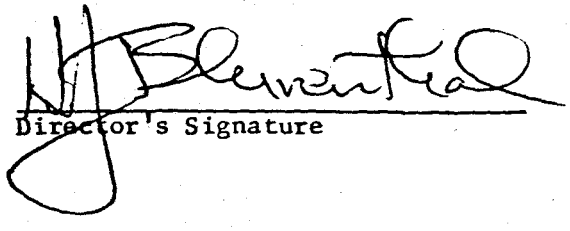
The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

12/21/76
Date


Director's Signature